PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A01N 1/02, 63/00, A61K 37/02, B01D 61/42, C12M 1/00, 1/02, 1/12, 1/36, C12N 5/16, 13/00

A1

(11) International Publication Number: WO 94/21117

(43) International Publication Date: 29 September 1994 (29.09.94)

(21) International Application Number: PCT/US94/03189

(22) International Filing Date: 23 March 1994 (23.03.94)

(30) Priority Data:
035,467 23 March 1993 (23.03.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US Not furnished (CIP)
Filed on Not furnished

(71) Applicant *(for all designated States except US)*: CBR LABORATORIES, INC. [US/US]; 800 Huntington Avenue, Boston, MA 02115 (US).

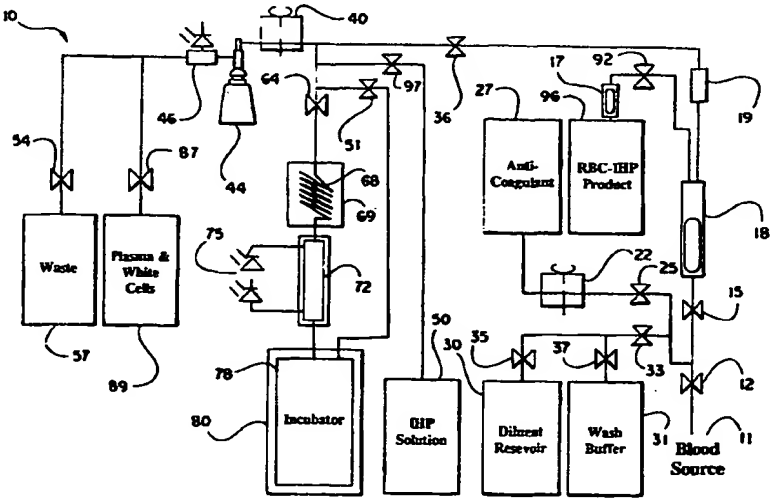
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NICOLAU, Yves, Claude [FR/US]; 54 LaGrange Street, Chestnut Hill, MA 02467 (US). BRUGGEMANN, Ulrich [DE/US]; 5 Arlington Street, Apartment 44, Cambridge, MA 02140 (US).

(74) Agents: TAUTVYDAS, Daiva, K. et al.; Jones & Askew, 37th floor, 191 Peachtree Street, N.E., Atlanta, GA 30303-1769 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD AND APPARATUS FOR ENCAPSULATION OF BIOLOGICALLY-ACTIVE SUBSTANCES IN CELLS



(57) Abstract

The present invention relates to a method and apparatus for the encapsulation of biologically-active substances in red blood cells, characterized by an optionally automated, continuous-flow self-contained electroporation system (10), as shown in the figure which allows withdrawal of blood from a patient (11), separation of red blood cells (44), encapsulation of a biologically-active substance in the cells (72), and optional recombination of blood plasma and the resulting modified red blood cells (96), thereby producing blood with modified biological characteristics. The present invention is particularly suited for use to encapsulate allosteric effectors of hemoglobin, thereby reducing the affinity of erythrocytes for oxygen and improving the release of oxygen from erythrocytes in tissues.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

"METHOD AND APPARATUS FOR ENCAPSULATION OF BIOLOGICALLY-ACTIVE SUBSTANCES IN CELLS"

Cross-Reference to Related Applications

This application is a continuation-in-part of U. S. Patent Application Serial Number 08/035,467, which is hereby incorporated by reference.

Technical Field

The present invention relates to methods and apparatuses for the encapsulation of biologically-active substances in various cell populations. More particularly, the present invention relates to a method and apparatus for the encapsulation of allosteric effectors of hemoglobin in erythrocytes by electroporation to achieve therapeutically desirable changes in the physical characteristics of the intracellular hemoglobin.

Background of the Invention

In the vascular system of an adult human being, blood has a volume of about 5 to 6 liters. Approximately one half of this volume is occupied by cells, including red blood cells (erythrocytes), white blood cells (leukocytes), and blood platelets. Red blood cells comprise the majority of the cellular components of blood. Plasma, the liquid portion of blood, is approximately

90 percent water and 10 percent various solutes. These solutes include plasma proteins, organic metabolites and waste products, and inorganic compounds.

The major function of red blood cells is to transport oxygen from the lungs to the tissues of the body, and transport carbon dioxide from the tissues to the lungs for removal. Very little oxygen is transported by the blood plasma because oxygen is only sparingly soluble in aqueous solutions. Most of the oxygen carried by the blood is transported by the hemoglobin of the erythrocytes. Erythrocytes in mammals do not contain nuclei, mitochondria or any other intracellular organelles, and they do not use oxygen in their own metabolism. Red blood cells contain about 35 percent by weight hemoglobin, which is responsible for binding and transporting oxygen.

Hemoglobin is a protein having a molecular weight of approximately 64,500. It contains four polypeptide chains and four heme prosthetic groups in which iron atoms are bound in the ferrous state. Normal globin, the protein portion of the hemoglobin molecule, consists of two α chains and two ß chains. Each of the four chains has a characteristic tertiary structure in which the chain is folded. The four polypeptide chains fit together in an approximately tetrahedral arrangement, to constitute the characteristic quaternary structure of hemoglobin. There is one heme group bound to each polypeptide chain which can reversibly bind one molecule of molecular oxygen. When hemoglobin combines with oxygen, oxyhemoglobin is formed. When oxygen is released, the oxyhemoglobin is reduced to deoxyhemoglobin.

Delivery of oxygen to tissues depends upon a number of factors including, but not limited to, the volume of blood flow, the number of red blood cells, the concentration of hemoglobin in the red blood cells, the oxygen affinity of the hemoglobin and, in certain species, on the molar ratio of intraerythrocytic hemoglobins with high and low oxygen affinity. The oxygen affinity of hemoglobin depends on four factors as well, namely: (1) the partial pressure of oxygen; (2) the pH; (3) the

concentration of 2,3-diphosphoglycerate (DPG) in the hemoglobin; and (4) the concentration of carbon dioxide. In the lungs, at an oxygen partial pressure of 100 mm Hg, approximately 98% of circulating hemoglobin is saturated with oxygen. This represents the total oxygen transport capacity of the blood. When fully oxygenated, 100 ml of whole mammalian blood can carry about 21 ml of gaseous oxygen.

The effect of the partial pressure of oxygen and the pH on the ability of hemoglobin to bind oxygen is best illustrated by examination of the oxygen saturation curve of hemoglobin. An oxygen saturation curve plots the percentage of total oxygen-binding sites of a hemoglobin molecule that are occupied by oxygen molecules when solutions of the hemoglobin molecule are in equilibrium with different partial pressures of oxygen in the gas phase.

The oxygen saturation curve for hemoglobin is sigmoid. Thus, binding the first molecule of oxygen increases the affinity of the remaining hemoglobin for binding additional oxygen molecules. As the partial pressure of oxygen is increased, a plateau is approached at which each of the hemoglobin molecules is saturated and contains the upper limit of four molecules of oxygen.

The reversible binding of oxygen by hemoglobin is accompanied by the release of protons, according to the equation:

$$HHb^+ + O_2 \rightleftharpoons HbO_2 + H^+$$

Thus, an increase in the pH will pull the equilibrium to the right and cause hemoglobin to bind more oxygen at a given partial pressure. A decrease in the pH will decrease the amount of oxygen bound.

In the lungs, the partial pressure of oxygen in the air spaces is approximately 90 to 100 mm Hg and the pH is also high relative to normal blood pH (up to 7.6). Therefore, hemoglobin will tend to become almost maximally saturated with oxygen in the lungs. At that pressure and pH, hemoglobin is approximately 98 percent saturated with oxygen. On the other hand, in the

capillaries in the interior of the peripheral tissues, the partial pressure of oxygen is only about 25 to 40 mm Hg and the pH is also relatively low (about 7.2 to 7.3). Because muscle cells use oxygen at a high rate thereby lowering the local concentration of oxygen, the release of some of the bound oxygen to the tissue is favored. As the blood passes through the capillaries in the muscles, oxygen will be released from the nearly saturated hemoglobin in the red blood cells into the blood plasma and thence into the muscle cells. Hemoglobin will release about a third of its bound oxygen as it passes through the muscle capillaries, so that when it leaves the muscle, it will be only about 64 percent saturated. In general, the hemoglobin in the venous blood leaving the tissue cycles between about 65 and 97 percent saturation with oxygen in its repeated circuits between the lungs and the peripheral tissues. Thus, oxygen partial pressure and pH function together to effect the release of oxygen by hemoglobin

A third important factor in regulating the degree of oxygenation of hemoglobin is the allosteric effector 2,3-diphosphoglycerate (DPG). DPG is the normal physiological effector of hemoglobin in mammalian erythrocytes. DPG regulates the oxygen-binding affinity of hemoglobin in the red blood cells in relationship to the oxygen partial pressure in the lungs. The higher the concentration of DPG in the cell, the lower the affinity of hemoglobin for oxygen.

When the delivery of oxygen to the tissues is chronically reduced, the concentration of DPG in the erythrocytes is higher than in normal individuals. For example, at high altitudes the partial pressure of oxygen is significantly less. Correspondingly, the partial pressure of oxygen in the tissues is less. Within a few hours after a normal human subject moves to a higher altitude, the DPG level in the red blood cells increases, causing more DPG to be bound and the oxygen affinity of the hemoglobin to decrease. Increases in the DPG level of red cells also occur in patients suffering from hypoxia. This adjustment allows the hemoglobin to release its bound oxygen more readily to the tissues to compensate for the decreased oxygenation of

hemoglobin in the lungs. The reverse change occurs when people acclimated to high altitudes and descend to lower altitudes.

As normally isolated from blood, hemoglobin contains a considerable amount of DPG. When hemoglobin is "stripped" of its DPG, it shows a much higher affinity for oxygen. When DPG is increased, the oxygen binding affinity of hemoglobin decreases. A physiologic allosteric effector such as DPG is therefore essential for the normal release of oxygen from hemoglobin in the tissues.

While DPG is the normal physiologic effector of hemoglobin in mammalian red blood cells, phosphorylated inositols are found to play the same role in the erythrocytes of some birds and reptiles. Although IHP is unable to pass through the mammalian erythrocyte membrane, it is capable of combining with hemoglobin of mammalian red blood cells at the binding site of DPG to modify the allosteric conformation of hemoglobin, the effect of which is to reduce the affinity of hemoglobin for oxygen. For example, DPG can be replaced by inositol hexaphosphate (IHP), which is even more potent than DPG in reducing the oxygen affinity of hemoglobin. IHP has a 1000-fold higher affinity to hemoglobin than DPG (R.E. Benesch et al., *Biochemistry*, Vol. 16, pages 2594-2597 (1977)) and increases the P50 of hemoglobin up to values of 96.4 mm Hg at pH 7.4 , and 37 degrees C (*J. Biol. Chem.*, Vol. 250, pages 7093-7098 (1975)).

The oxygen release capacity of mammalian red blood cells can be enhanced by introducing certain allosteric effectors of hemoglobin into erythrocytes, thereby decreasing the affinity of hemoglobin for oxygen and improving the oxygen economy of the blood. This phenomenon suggests various medical applications for treating individuals who are experiencing lowered oxygenation of their tissues due to the inadequate function of their lungs or circulatory system.

Because of the potential medical benefits to be achieved from the use of these modified erythrocytes, various techniques have been developed in the prior art to enable the encapsulation of allosteric effectors of hemoglobin in erythrocytes.

Accordingly, numerous devices have been designed to assist or simplify the encapsulation procedure. The encapsulation methods known in the art include osmotic pulse (swelling) and reconstitution of cells, controlled lysis and resealing, incorporation of liposomes, and electroporation. Current methods of electroporation make the procedure commercially impractical on a scale suitable for commercial use.

The following references describe the incorporation of polyphosphates into red blood cells by the interaction of liposomes loaded with IHP: *Gersonde, et al.*, "Modification of the Oxygen Affinity of Intracellular Haemoglobin by Incorporation of Polyphosphates into Intact Red Blood Cells and Enhanced O2 Release in the Capillary System", *Biblthca. Haemat.*, No. 46, pp. 81-92 (1980); *Gersonde, et al.*, "Enhancement of the O2 Release Capacity and of the Bohr-Effect of Human Red Blood Cells after Incorporation of Inositol Hexaphosphate by Fusion with Effector-Containing Lipid Vesicles", *Origins of Cooperative Binding of Hemoglobin*, (1982); and Weiner, "Right Shifting of Hb-O_2 Dissociation in Viable Red Cells by Liposomal Technique," *Biology of the Cell*, Vol. 47, (1983).

Additionally, U.S. Patent Nos. 4,192,869, 4,321,259, and 4,473,563 to *Nicolau et al.* describe a method whereby fluid-charged lipid vesicles are fused with erythrocyte membranes, depositing their contents into the red blood cells. In this manner it is possible to transport allosteric effectors such as inositol hexaphosphate into erythrocytes, where, due to its much higher binding constant IHP replaces DPG at its binding site in hemoglobin.

In accordance with the liposome technique, IHP is dissolved in a phosphate buffer until the solution is saturated and a mixture of lipid vesicles is suspended in the solution. The suspension is then subjected to ultrasonic treatment or an injection process, and then centrifuged. The upper suspension contains small lipid vesicles containing IHP, which are then collected. Erythrocytes are added to the collected suspension and incubated, during which time the lipid vesicles containing IHP fuse with the cell

membranes of the erythrocytes, thereby depositing their contents into the interior of the erythrocyte. The modified erythrocytes are then washed and added to plasma to complete the product.

The drawbacks associated with the liposomal technique include poor reproducibility of the IHP concentrations incorporated in the red blood cells and significant hemolysis of the red blood cells following treatment. Additionally, commercialization is not practical because the procedure is tedious and complicated.

In an attempt to solve the drawbacks associated with the liposomal technique, a method of lysing and the resealing red blood cells was developed. This method is described in the following publication: *Nicolau, et al.*, "Incorporation of Allosteric Effectors of Hemoglobin in Red Blood Cells. Physiologic Effects," *Biblthca. Haemat.*, No. 51, pp. 92-107, (1985). Related U.S. Patent Nos. 4,752,586 and 4,652,449 to *Ropars et al.* also describe a procedure of encapsulating substances having biological activity in human or animal erythrocytes by controlled lysis and resealing of the erythrocytes, which avoids the RBC-liposome interactions.

The technique is best characterized as a continuous flow dialysis system which functions in a manner similar to the osmotic pulse technique. Specifically, the primary compartment of at least one dialysis element is continuously supplied with an aqueous suspension of erythrocytes while the secondary compartment of the dialysis element contains an aqueous solution which is hypotonic with respect to the erythrocyte suspension. The hypotonic solution causes the erythrocytes to lyse. The erythrocyte lysate is then contacted with the biologically active substance to be incorporated into the erythrocyte. To reseal the membranes of the erythrocytes, the osmotic and/or oncotic pressure of the erythrocyte lysate is increased and the suspension of resealed erythrocytes is recovered.

In related U.S. Patent Nos. 4,874,690 and 5,043,261 to *Goodrich et al.* a related technique involving lyophilization and reconstitution of red blood cells is disclosed. As part of the

process of reconstituting the red blood cells, the addition of various polyanions, including inositol hexaphosphate, is described. Treatment of the red blood cells according to the process disclosed results in a cell with unaffected activity. Presumably, the IHP is incorporated into the cell during the reconstitution process, thereby maintaining the activity of the hemoglobin.

In U.S. Patent Nos. 4,478,824 and 4,931,276 to *Franco et al.* a second related method and apparatus is described for introducing effectively non-ionic agents, including inositol hexaphosphate, into mammalian red blood cells by effectively lysing and resealing the cells. The procedure is described as the "osmotic pulse technique." In practicing the osmotic pulse technique, a supply of packed red blood cells is suspended and incubated in a solution containing a compound which readily diffuses into and out of the cells, the concentration of the compound being sufficient to cause diffusion thereof into the cells so that the contents of the cells become hypertonic. Next, a trans-membrane ionic gradient is created by diluting the solution containing the hypertonic cells with an essentially isotonic aqueous medium in the presence of at least on desired agent to be introduced, thereby causing diffusion of water into the cells with a consequent swelling and an increases in permeability of the outer membranes of the cells. This "osmotic pulse" causes the diffusion of water into the cells and a resultant swelling of the cells which increase the permeability of the outer cell membrane to the desired agent. The increase in permeability of the membrane is maintained for a period of time sufficient only to permit transport of least one agent into the cells and diffusion of the compound out of the cells.

Polyanions which may be used in practicing the osmotic pulse technique include pyrophosphate, tripolyphosphate, phosphorylated inositols, 2,3-diphosphoglycerate (DPG), adenosine triphosphate, heparin, and polycarboxylic acids which are water-soluble, and non-disruptive to the lipid outer bilayer membranes of red blood cells.

The osmotic pulse technique has several shortcomings including low yield of encapsulation, incomplete resealing, lose of cell content and a corresponding decrease in the life span of the cells. The technique is tedious, complicated and unsuited to automation. For these reasons, the osmotic pulse technique has had little commercial success.

Another method for encapsulating various biologically-active substances in erythrocytes is electroporation. Electroporation has been used for encapsulation of foreign molecules in different cell types including IHP red blood cells as described in *Mouneimne, et al.*, "Stable rightward shifts of the oxyhemoglobin dissociation curve induced by encapsulation of inositol hexaphosphate in red blood cells using electroporation," FEBS, Vol. 275, No. 1, 2, pp. 117-120 (1990).

The process of electroporation involves the formation of pores in the cell membranes, or in any vesicles, by the application of electric field pulses across a liquid cell suspension containing the cells or vesicles. During the poration process, cells are suspended in a liquid media and then subjected to an electric field pulse. The medium may be electrolyte, non-electrolyte, or a mixture of electrolytes and non-electrolytes. The strength of the electric field applied to the suspension and the length of the pulse (the time that the electric field is applied to a cell suspension) varies according to the cell type. To create a pore in a cell's outer membrane, the electric field must be applied for such a length of time and at such a voltage as to create a set potential across the cell membrane for a period of time long enough to create a pore.

Four phenomenon appear to play a role in the process of electroporation. The first is the phenomenon of dielectric breakdown. Dielectric breakdown refers to the ability of a high electric field to create a small pore or hole in a cell membrane. Once a pore is created, a cell can be loaded with a biologically-active substances. The second phenomenon is the dielectric bunching effect, which refers to the mutual self attraction produced by the placement of vesicles in a uniform electric field.

The third phenomenon is that of vesicle fusion. Vesicle fusion refers to the tendency of membranes of biological vesicles, which have had pores formed by dielectric breakdowns, to couple together at their mutual dialectic breakdown sites when they are in close proximity. The fourth phenomenon is the tendency of cells to line up along one of their axis in the presence of high frequency electric fields. Thus, electroporation relates to the use in vesicle rotational prealignment, vesicle bunching and dielectric constant or vesicles for the purpose of loading and unloading the cell vesicle.

Electroporation has been used effectively to incorporate allosteric effectors of hemoglobin in erythrocytes. In an article by *Mouneimne, Y et al.*, "Stable Rightward Shifts of Oxyhemoglobin Disassociation Constant Induced by Encapsulation of Inositol Hexaphosphate in Red Blood Cells Using Electroporation", FEBS, Vol. 275, No. 1, 2, pages 11-120. *Mouneimne* and his colleagues reported that right shifts of the hemoglobin-oxygen dissociation in treated erythrocytes having incorporated IHP can be achieved. Measurements at 24 and 48 hours after loading with IHP showed a stable P_{50} value indicating that resealing of the erythrocytes was permanent. Furthermore, it was shown that red blood cells loaded with inositol hexaphosphate have a normal half life of eleven days. However, the results obtained by *Mouneimne* and his colleagues indicate that approximately 20% of the retransfused cells were lost within the first 24 hours of transfusion.

The electroporation methods disclosed in the prior art are not suitable for processing large volumes of sample, nor use of a high or repetitive electric charge. Furthermore, the methods are not suitable for use in a continuous or "flow" electroporation chamber. Available electroporation chambers are designed for static use only. Namely, processing of samples by batch. Continuos use of a "static" chamber results in over heating of the chamber and increased cell lysis. Furthermore, the existing technology is unable to incorporate a sufficient quantity of IHP in a sufficient percentage of the cells being processed to dramatically

change the oxygen carrying capacity of the blood. In addition, the prior art methods require elaborate equipment and are not suited for loading red blood cells of a patient on site. Thus, the procedure is time consuming and not suitable for use on a commercial scale. What is needed is a simple, efficient and rapid method for encapsulating biologically-active substances in erythrocytes while preserving the integrity and biologic function of the cells. The potential therapeutic applications of biologically altered blood cells suggests the need for simpler, and more effective and complete methods of encapsulation of biologically-active substances, including allosteric effectors of hemoglobin in intact erythrocytes.

There are numerous clinical conditions that would benefit from treatments that would increase tissue delivery of oxygen bound to hemoglobin. For example, the leading cause of death in the United States today is cardiovascular disease. The acute symptoms and pathology of many cardiovascular diseases, including congestive heart failure, myocardial infarction, stroke, intermittent claudication, and sickle cell anemia, result from an insufficient supply of oxygen in fluids that bathe the tissues. Likewise, the acute loss of blood following hemorrhage, traumatic injury, or surgery results in decreased oxygen supply to vital organs. Without oxygen, tissues at sites distal to the heart, and even the heart itself, cannot produce enough energy to sustain their normal functions. The result of oxygen deprivation is tissue death and organ failure.

Although the attention of the American public has long been focused on the preventive measures required to alleviate heart disease, such as exercise, appropriate dietary habits, and moderation in alcohol consumption, deaths continue to occur at an alarming rate. Since death results from oxygen deprivation, which in turn results in tissue destruction and/or organ dysfunction, one approach to alleviate the life-threatening consequences of cardiovascular disease is to increase oxygenation of tissues during acute stress. The same approach is also

appropriate for persons suffering from blood loss or chronic hypoxic disorders, such as congestive heart failure.

Another condition which could benefit from an increase in the delivery of oxygen to the tissues is anemia. A significant portion of hospital patients experience anemia or a low "crit" caused by an insufficient quantity of red blood cells or hemoglobin in their blood. This leads to inadequate oxygenation of their tissues and subsequent complications. Typically, a physician can temporarily correct this condition by transfusing the patient with units of packed red blood cells.

Enhanced blood oxygenation may also reduce the number of heterologous transfusions and allow use of autologous transfusions in more case. The current method for treatment of anemia or replacement of blood loss is transfusion of whole human blood. It is estimated that three to four million patients receive transfusions in the U.S. each year for surgical or medical needs. In situations where there is more time it is advantageous to completely avoid the use of donor or heterologous blood and instead use autologous blood.

Often the amount of blood which can be drawn and stored prior to surgery limits the use of autologous blood. Typically, a surgical patient does not have enough time to donate a sufficient quantity of blood prior to surgery. A surgeon would like to have several units of blood available. As each unit requires a period of several weeks between donations and can not be done less than two weeks prior to surgery, it is often impossible to sequester an adequate supply of blood. By processing autologous blood with IHP, less blood is required and it becomes possible to completely avoid the transfusion of heterologous blood.

As IHP-treated red cells transport 2-3 times as much oxygen as untreated red cells, in many cases, a physician will need to transfuse fewer units of IHP-treaded red cells. This exposes the patient to less heterologous blood, decreases the extent of exposure to viral diseases from blood donors and minimizes immune function disturbances secondary to transfusions. The ability to infuse more efficient red blood cells is also

advantageous when the patients blood volume is excessive. In other more severe cases, where oxygen transport is failing, the ability to rapidly improve a patient's tissue oxygenation is life saving.

Although it is evident that methods of enhancing oxygen delivery to tissues have potential medical applications, currently there are no methods clinically available for increasing tissue delivery of oxygen bound to hemoglobin. Transient, 6 to 12 hour elevations of oxygen deposition have been described in experimental animals using either DPG or molecules that are precursors of DPG. The natural regulation of DPG synthesis in vivo and its relatively short biological half-life, however, limit the DPG concentration and the duration of increased tissue PO_2, and thus limit its therapeutic usefulness.

Additionally, as reported in Genetic Engineering News, Vol. 12, No. 6, April 15, 1992, several groups are attempting to engineer free oxygen-carrying hemoglobin as a replacement for human blood. Recombinant, genetically modified human hemoglobin that does not break down in the body and that can readily release up to 30% of its bound oxygen is currently being tested by Somatogen, Inc., of Boulder Colorado. While this product could be useful as a replacement for blood lost in traumatic injury or surgery, it would not be effective to increase Po_2 levels in ischemic tissue, since its oxygen release capacity is equivalent to that of natural hemoglobin (27-30%). As are all recombinant products, this synthetic hemoglobin is also likely to be a costly therapeutic.

Synthetic human hemoglobin has also been produced in neonatal pigs by injection of human genes that control hemoglobin production. This may be a less expensive product than the Somatogen synthetic hemoglobin, but problems with oxygen affinity and breakdown of hemoglobin in the body are not solved by the method.

What is needed is a simple, efficient and rapid method for encapsulating biologically-active substances, such as IHP, in erythrocytes without damaging the erythrocytes.

Summary of the Invention

The present invention relates to a method and apparatus for the encapsulation of biologically-active substances in various cell populations. More specifically, the present invention provides an automated, self-contained, flow apparatus for encapsulating allosteric effectors, such as inositol hexaphosphate, in red blood cells, thereby reducing the affinity of the hemoglobin for oxygen and enhancing the delivery of oxygen by red blood cells to tissues. Encapsulation is preferrably achieved by electroporation; however, it is contemplated that other methods of encapsulation may be used in practicing the present invention. The method and apparatus of the present invention is equally suited to the encapsulation of a variety of biologically-active substances in various cell populations.

The apparatus and method of the present invention is suited to the incorporation of a variety of biologically-active substances in cells and lipid vesicles. The method and apparatus of the present invention may be used for introducing a compound or biologically-active substance into a vesicle whether that vesicle is engineered or naturally occurring. For example, the apparatus and method of the present invention may be used to introduce IHP into erythrocytes.

The encapsulation of inositol hexaphosphate in red blood cells by electroporation according to the present invention results in a significant decrease in the hemoglobin affinity for oxygen without affecting the life span, ATP levels, K+ levels, or normal rheological competence of the cells. In addition, the Bohr effect is not altered except to shift the O_2 binding curve to the right. Lowering the oxygen affinity of the erythrocytes increases the capacity of erythrocytes to dissociate the bound oxygen and thereby improves the oxygen supply to the tissues. Enhancement of the oxygen-release capacity of erythrocytes brings about significant physiological effects such as a reduction in cardiac

output, an increase in the arteriovenous differences, and improved tissue oxygenation.

The modified erythrocytes prepared in accordance with the present invention, having improved oxygen release capacities, may find their use in situations such as those illustrated below:

1. Under conditions of low oxygen-partial pressure, such as at high altitudes;
2. When the oxygen exchange surface of the lung is reduced, such as occurs in emphysema;
3. When there is an increased resistance to oxygen diffusion in the lung, such as occurs in pneumonia or asthma;
4. When there is a decrease in the oxygen-transport capacity of erythrocytes, such as occurs with erythropenia or anemia, or when an arteriovenous shunt is used;
5. To treat blood circulation disturbances, such as arteriosclerosis, thromboembolic processes, organ infarct or ischemia;
6. To treat conditions of high oxygen affinity of hemoglobin, such as hemoglobin mutations, chemical modifications of N-terminal amino acids in the hemoglobin-chains, or enzyme defects in erythrocytes;
7. To accelerate detoxification processes by improving oxygen supply;
8. To decrease the oxygen affinity of conserved blood; or
9. To improve the efficacy of various cancer treatments.

According to the method and apparatus of the present invention, it is possible to produce modified erythrocytes which contribute to an improved oxygen economy of the blood. These modified erythrocytes are obtained by incorporation of allosteric effectors, such as IHP, by electroporation of the erythrocyte membranes.

The incorporation of the biologically-active substances into the cells in accordance with the method of the present invention, including the encapsulation of allosteric effectors of hemoglobin into erythrocytes, is conducted extracorporally via an automated, flow electroporation apparatus. Briefly, a cell suspension is introduced into the separation and wash bowl chamber of the flow encapsulation apparatus. The cells are separated from the suspension, washed and resuspended in a solution of the biologically-active substance to be introduced into the cell. This suspension is introduced into the electroporation chamber and then incubated. Following electroporation and incubation, the cells are washed and separated. A contamination check is optionally conducted to confirm that all unencapsulated biologically-active substance has been removed. Then, the cells are prepared for storage or reintroduction into a patient.

In accordance with the present invention and with reference to the preferred embodiment, blood is drawn from a patient, the erythrocytes are separated from the drawn blood, the erythrocytes are modified by the incorporation of allosteric effectors and the modified erythrocytes and blood plasma is reconstituted. In this manner, it is possible to prepare and store blood containing IHP-modified erythrocytes.

The apparatus of the present invention provides an improved method for the encapsulation of biologically-active substances in cells including an apparatus which is self-contained and therefore sterile, an apparatus which can process large volumes of cells within a shortened time period, an apparatus having improved contamination detection, cooling and incubation elements, an apparatus is entirely automated and which does not require the supervision of a technician once a sample is introduced into the apparatus.

Thus, it is an object of the present invention to provide an automated, continuous flow encapsulation apparatus.

It is a further object of the present invention to provide an automated, continuous flow electroporation apparatus.

It is a further object of the present invention to provide a continuous flow encapsulation apparatus which produces a homogenous population of loaded cells or vesicles.

It is another object of the present invention to provide a continuous flow electroporation device which produces a homogenous population of loaded cells or vesicles.

It is another object of the present invention to provide a sterile and nonpyrogenic method of encapsulating biologically-active substances in cells.

It is another object of the present invention to provided a method and apparatus which results in stable resealing of cells or vesicles following electroporation to minimize lysis of the modified cells or vesicles after electroporation.

It is another object of the present invention to provide a flow encapsulation apparatus which produces a modified cell population from which all exogenous non-encapsulated biologically-active substances have been removed.

It is another object of the present invention to provide an electroporation apparatus which produces a modified cell population from which all exogenous, non-encapsulated biologically-active substances have been removed.

It is another object of the present invention to provide a method and apparatus that allows continuous encapsulation of biologically-active substances in a population of cells or vesicles.

It is a further object of the present invention to provide a method and apparatus that achieves the above-defined objects, features, and advantages in a single cycle.

It is another object of the present invention to provide a continuous flow electroporation chamber.

It is another object of the present invention to provide an improved and more efficient method of encapsulating biologically active substances in cells than those methods currently available.

It is a further object of the present invention to provide a population of artificial cells suitable for medical use.

It is a further object of the present invention to provide a composition suitable for use in the treatment of conditions and/or disease states resulting from a lack of or decrease in oxygenation.

Other objects, features, and advantages of the present invention will become apparent upon reading the following detailed description of the preferred embodiment of the invention when taken in conjunction with the drawing and the appended claims.

Brief Description of the Drawings

Fig. 1 is a schematic diagram of a first embodiment of a continuous flow encapsulation apparatus.

Fig. 2 is a schematic diagram of a second embodiment of a continuous flow encapsulation apparatus.

Fig. 3 is a top view of a first embodiment of the flow electroporation chamber with electrodes.

Fig. 4 is a top view of a first embodiment of the flow electroporation chamber without electrodes.

Fig. 5 is a side view of a first embodiment of the flow electroporation chamber.

Fig. 6 is an end view of a first embodiment of the flow electroporation chamber.

Fig. 7 is a side view of an electrode for use with the first embodiment of the flow electroporation chamber.

Fig. 8 is a front view of the electrode of Fig. 7.

Fig. 9 is an exploded perspective view of a second embodiment of the flow electroporation chamber.

Fig 10 is a perspective view of the flow electroporation chamber of Fig. 9 with the chamber being assembled.

Fig. 11 is a graph comparing the effect of various fieldstrengths, under static or flow conditions, on the % oxygenation of IHP-encapsulated red blood cells.

Fig. 12 is a table comparing the effects of various fieldstrengths, under static or flow conditions, on the P_{50} value of IHP-encapsulated red blood cells .

Fig. 13 is a table comparing the survival rates of red blood cells subjected to electroporation under static and flow conditions at various fieldstrengths.

Detailed Description of the Invention

The present invention provides an automated, self-contained, flow apparatus for encapsulating allosteric effectors, such as inositol hexaphosphate, in red blood cells. The apparatus of the present invention combines the features of a plasmaphoresis apparatus with those of a flow electroporation apparatus to form an automated, self-contained flow electroporation device. The present invention further comprises a new flow electroporation chamber that allows use of the chamber under flow rather than static conditions. It is contemplated that the method and apparatus of the present invention may be used to encapsulate a variety of biologically-active substances in diverse cell populations.

Additionally, the present invention provides a population of modified cells having physical characteristics that make the cells particularly useful for treating conditions which demand or benefit from an increase in the delivery of oxygen to the tissues. In accordance with the method of the present invention, a homogenous population of IHP loaded red blood cells can be obtained with reduced contamination and a reduced propensity to lyse following encapsulation. The treated red blood cells exhibit normal life spans in circulation. Using the present invention, red blood cells of a patient in need of the treatment can be quickly loaded and returned to the patient's circulation.

The method of operation of the apparatus of the present invention is described below with reference to the preferred use of the apparatus, i.e., the encapsulation of allosteric effectors of hemoglobin in red blood cells. Inositol hexaphosphate is the preferred allosteric effector to be used with the present invention. Other sugar phosphates, such as inositol pentaphosphate, inositol tetraphosphate, inositol triphosphate, inositol diphosphate and diphosphatidyl inositol diphosphate, can also be used. Other

suitable allosteric effectors include polyphosphates such as nucleotide triphosphates, nucleotide diphosphates, nucleotide monophosphates, and alcohol phosphate esters. In case of certain mutations of hemoglobin, e.g. "Zurich" hemoglobin, organic anions such as polycarboxylic acids can be used as allosteric effectors. Finally, it is possible to use inorganic anions such as hexacyano ferrate, phosphate or chloride as allosteric effectors.

Red blood cells that have been loaded with inositol hexaphosphate according to the present invention can be used to treat a wide variety of diseases and disease states. The IHP loaded red blood cells made according to the present invention can be administered to a patient undergoing a heart attack thereby increasing the oxygen delivery to the ischemic heart tissue and, at the same time, reducing the cardiac output. The IHP-loaded red blood cells made according to the present invention also can be used to treat any ischemic condition including, but not limited to, stroke, diabetes, sickle cell disease, burns, intermittent claudication, emphysema, hypothermia, peripheral vascular disease, congestive heart failure, angina, transient ischemic disease, disseminated intravascular coagulation, adult respiratory distress syndrome (ARDS) and cystic fibrosis. A detailed description of the medical applications of compositions prepared in accordance with the method of the present invention is also provided below.

Continuous Flow Encapsulation Apparatus

The method of operation of the apparatus of the present invention is described below with reference to the preferred use of the apparatus, i.e., the encapsulation of allosteric effectors of hemoglobin in red blood cells by electroporation. It is to be understood that the apparatus may be adapted to accommodate other cell populations or vesicles, and other biologically active substances. Additionally, the apparatus maybe adapted to utilize methods of encapsulation other than electroporation.

Briefly, in accordance with the present invention, a sample of blood is introduced into the continuos flow encapsulation

apparatus. If red blood cells are being collected, the blood can either be drawn directly from a patient or can be previously drawn blood. The blood is initially separated into red blood cells, plasma and white blood cells, and waste products. The waste products include the diluent and various blood solutes remaining in the supernatant after centrifugation. They are stored in a waste reservoir within the apparatus. The blood plasma and white blood cells are also retained in a reservoir within the system while the red blood cells are admixed with the substance to be encapsulated. The suspension of red blood cells is then subjected to electroporation. Following electroporation, the red blood cells are incubated under conditions which allow the cells to reseal. They are then processed and washed to eliminate exogenous, non-encapsulated biologically-active substances. When the cells have been processed, the red blood cells containing the encapsulated substances can be optionally reconstituted with the blood plasma and white blood cells. The reconstituted blood may then be returned directly to the patient or can be stored for later use. Although described as discrete steps, the process is essentially continuos.

A first embodiment of the present invention is described with reference to Fig. 1, which schematically illustrates the structure of the continuous flow encapsulation apparatus of the present invention.

In accordance with the present invention, a volume of whole blood is admitted into the electroporation system **5** at input **11**. The blood sample may optionally be drawn directly from a patient into the electroporation system **5**, or the blood may be drawn at an earlier time and stored prior to introduction into the system **5**. Valve **12** is opened to admit the sample into the system **5**. Simultaneously, valve **25** is opened and pump **22** is engaged to admit an anti-coagulant from the anti-coagulant reservoir **27**. A suitable anticoagulant is heparin, although other anticoagulants can be used. The preferred anticoagulant is ACD. Valves **15** and **36** are also opened and pump **40** is engaged. The admixture of anticoagulant and whole blood passes through a filter **18** and a

pressure evaluation system **19** that monitors the flow through the apparatus, and is collected in a blood separation and wash bowl **44** which is activated when pump **40** is engaged. A sensor indicates when the blood separation and wash bowl **44** has been filled with red blood cells. When it has been filled, they blood supply is stopped. The steps involving separation of the blood components can be accomplished by a plasmaphoresis apparatus, such as the plasmaphoresis apparatus manufactured by Haemonetics Corporation (Haemonetics Corporation, Braintree, MA).

As explained above, when pump **40** is engaged in a clockwise direction, the blood separation and wash bowl **44** is engaged and the anti-coagulant and whole blood suspension is centrifuged to separate the plasma, white blood cells, red blood cells, and waste. Valve **87** is opened to admit the plasma and white blood cells into the plasma reservoir **89**.

Optionally and dependent on the cell population being processed by the apparatus, the cells retained in the blood separation and was bowl **44** are then washed. Valves **33, 15,** and **36** are opened to admit saline buffer from the diluent reservoir **30** into the blood separation and wash bowl **44** which contains the red blood cells. Pump **40** is still engaged. The red blood cells are then washed and centrifuged. The preferred saline buffer is a .9% sodium chloride solution, although other physiologically isotonic buffers can be used to dilute and wash the red blood cells. Valve **54** is opened to admit the waste into the waste reservoir **57** during the washing process. Again, the waste is stored in the waste reservoir **57** and the red blood cells are retained in the blood separation and wash bowl **44**. The wash process is repeated if necessary.

Following separation of the red blood cells, pump **40** is reversed, pump **22** is turned off, valves **12, 15, 33, 36, 25, 87,** and **54** are closed, and valves **97** and **64** are opened. The IHP solution is pumped out of the IHP reservoir **50** while, simultaneously, red blood cells are pumped out of the blood separation and wash bowl **44** towards the cooling coil **68**. The

red blood cells and IHP solution are admixed in the tubing of the apparatus at junction **67** and then pumped through the cooling coil **68**. In a preferred embodiment of the present invention, and as explained in detail below, the IHP solution and red blood cells may be admixed in the separation and wash bowl **44** before being admitted into the cooling coil **68**.

The preferred concentration of IHP in the solution is between approximately 10 mMol and 100 mMol with a more preferred concentration of approximately 23 to 35 mMol, and a most preferred concentration of 35 mMol. The preferred IHP solution comprises the following compounds, in the following concentrations:

35 mMol IHP salt neutralized with 35 mMol IHP acid to a pH of 7.3
33 mMol K_2HPO_4
7.0 mMol NaH_2
30.6 mMol KCL
6.4 mMol NaCl
7.3 mMol Sucrose
5.0 mMol ATP

A second IHP solution for use with the present invention comprises the following compounds, in the following concentrations:

23 mMol IHP salt neutralized with HCl to a pH of 7.3
40 mMol K_2HPO_4
7 mMol NaH_2

The IHP may be obtained from Sigma Chemical Company of St. Louis, Missouri.

The hematocrit of the suspension is preferably between approximately 30 and 80 with the most preferred hematocrit of approximately 50. Pump **40** is designed to pump both red blood

cells and IHP solution and can be adjusted so that the final hematocrit entering the cooling coil **68** can be predetermined.

After mixing, the red blood cell-IHP suspension is then pumped through a cooling coil **68**. Cooling can be achieved with a water bath or with a thermo-electric based cooling system. For example, cooling coil **68** is immersed in a cooling bath in the cooling reservoir **69**. When the red blood cell-IHP suspension passes through the cooling coil **68**, the suspension is cooled to a temperature of between approximately 1°C and 12°C, preferably approximately 4°C. Cooling the red blood cells ensures the survival of the pore created in the cell membrane during the electroporation process. The use of a cooling coil aids in the speed of cooling by increasing the surface area of the sample in contact with the cooling element. Optionally, the cooling coil can be surrounded by a thermo-electric heat pump.

Certain applications may require heating of the cell suspension prior to electroporation. In such a case, a heating coil may replace the cooling coil **68**. The maximum temperature tolerated by red blood cells is approximately 37°C.

A thermoelectric heat pump works by extracting thermal energy from a particular region, thereby reducing its temperature, and then rejecting the thermal energy into a "heat sink" region of higher temperature. At the cold junction, energy is absorbed by electrons as they pass from a low energy level in the p-type semiconductor element, to a higher energy level in the n-type semiconductor element. The power supply provides the energy to move the electrons through the system. At the hot junction, energy is expelled into a heat sink as electrons move from a high energy level element (n-type) to a lower energy level element (p-type).

Thermoelectric elements are totally solid state and do not have moving mechanical parts or require a working fluid, as do vapor-cycle devices. However, thermoelectric heat pumps perform the same cooling functions as freon-based vapor compression or absorption refrigerators. Thermoelectric heat pumps are highly reliable, small in size and capacity, low cost,

low weight, intrinsically safer than many other cooling devices, and are capable of precise temperature control.

The preferred thermoelectric heat pumps for use in the present invention are manufactured by MELCOR Materials Electronic Products Corp. of Trenton, New Jersey. The thermocouples are made of high performance crystalline semiconductor material. The semiconductor material is bismuth telluride, a quaternary alloy of bismuth, tellurium, selenium, and antimony, doped and processed to yield oriented polycrystalline semiconductors with properties. The couples, connected in series electrically and in parallel thermally, are integrated into modules. The modules are packaged between metallized ceramic plates to afford optimum electrical insulation and thermal conduction with high mechanical strength in compression. Modules can be mounted in parallel to increase the heat transfer effect or can be stacked in mullet-stage cascades to achieve high differential temperatures. Passing a current through the heat pump generates a temperature differential across the thermocouples, with maximum ratings of 70° C and higher.

After cooling, the red blood cell-IHP suspension enters the electroporation chamber **72** where an electric pulse is administered from a pulse generator **75** to the red blood cell-IHP suspension, causing openings to form within the cell membranes of the red blood cells. Optionally, an automatic detection system will turn the pulse generator **75** on when the chamber **72** is filled with red blood cell-IHP suspension. An electrical pule is applied to the suspension every time the chamber **72** is filled with unencapsulated cells. A conventional electroporation chamber may be used when the operation of the apparatus is static, namely, when single discrete batches of cells are processed. In a preferred embodiment of the present invention a flow electroporation chamber is used. In one embodiment, a flow electroporation chamber **72** is constructed of clear polyvinyl chloride, and contains two opposing electrodes spaced a distance of 7 mm apart. The distance between the electrodes will vary depending on the flow volume and fieldstrength. Preferably, the

flow electroporation chamber **72** is disposable. The electroporation chamber may also be constructed of polysolfone, which is preferably for use with certain sterilization procedures, such as autoclaving. A detailed description of the structure and construction of the flow electroporation chamber is provided below.

The red blood cell-IHP suspension passes between the two electrodes of the electroporation chamber **72**. When a suspension of non-treated cells enter the chamber **72**, an electrical field of 1 to 3 KV/cm is created and maintained for a period of 1 to 4 milliseconds, preferably for a period of 2 milliseconds with a 1.8 ml flow chamber. Preferably, the IHP-red blood cell suspension is subjected to three high voltage pulses per volume at a fieldstrength of approximately 2600 to 3200 V/cm per pulse. The pulse of current across the cell membranes causes an electrical breakdown of the cell membranes, which creates pores in the membranes. IHP then diffuses into the cell through these pores.

Following electroporation, the red blood cell-IHP suspension enters an incubation chamber **78** where the suspension is incubated at room temperature for an incubation time of between approximately 15 minutes and 120 minutes with the preferred incubation time of 30 to 60 minutes. Optionally, the red blood cell-IHP suspension is incubated for approximately 5 minutes at a temperature of approximately 37° C, and at least 15 minutes at room temperature. The incubation chamber **78** may optionally be surrounded by a heating means **80**. For example, the heating means **80** can be a water bath or can be a thermoelectric heat pump.

Optionally, the incubator **78** contains a resealing buffer which aids in resealing and reconstitution of the red blood cells. The preferred composition of the resealing buffer is provided below:

RESEALING BUFFER

I. Combine	
Sodium chloride	150 mMol
Potassium chloride	8 mMol
Sodium phosphate	6 mMol
Magnesium sulfate	2 mMol
Glucose	10 mMol
Adenine	1 mMol
Inosine	1 mMol
Penicillin G	500 units/ml
Chloramphenicol	0.1mg/ml

II. Add	
BSA	3.5%
Calcium chloride	2 mMol

In the preferred embodiment of the present invention, no resealing buffer is used.

Following incubation, valve **51** is opened and pump **40** is engaged and the red blood cell-IHP suspension is returned to the blood separation and wash bowl **44** from the incubation chamber **78**. The excess IHP solution is removed from the red blood cell suspension by centrifugation. The waste IHP solution is directed to waste reservoir **57**. Valves **33, 15** and **36** are then opened to admit a volume of diluent into the blood separation and wash bowl **44**. The red blood cell-IHP suspension is then centrifuged and the supernatant is discarded in the waste reservoir **57** through valve **54** leaving the red blood cells in the blood separation and wash bowl **44**. A saline buffer is added to the modified red blood cells from the diluent reservoir **30**. The cells are washed and the supernatant is discarded following centrifugation. The wash process is repeated if needed.

Optionally, as the waste is removed from the separation and wash bowl **44** it passes through a contamination detector **46** to detect any free IHP in the waste solution thereby confirming that

exogenous non-encapsulated IHP has been removed from the modified red blood cells. The contamination detection system relies on optical changes in the washing buffer. After the modified red blood cells have been washed and centrifuged, the supernatant passes through the contamination detector **64** before it is deposited in the waste reservoir **57**. If exogenous, non-encapsulated IHP remains in the washing buffer, The discarded solution will be turbid. The turbidity is due to the reaction of IHP with calcium, which is a component of the wash buffer. The contamination detector **46** uses an optical detection system. Preferably, the light source is an LED and the detector is a photodiode. The voltage difference of the photodiode will indicate the amount of IHP in the wash solution. The contamination detector **46** is optional.

Following washing, the IHP-red blood cell product is optionally reconstituted with the plasma and white blood cells which had been retained in reservoir **89**. The treated red blood cells may be collected in a reinjection bag, either in a preservation media or in the autologous plasma of the patient.

The IHP-loaded red blood cells obtained can be administered directly back into the patient or the cells can be stored for later use. The IHP in the red blood cells is not released during the normal storage time.

A preferred embodiment of the present invention is described with reference to Fig. 2, which schematically illustrates the structure of the continuous flow encapsulation apparatus of the present invention. Again, the method of operation of the apparatus is described with reference to the preferred use of the apparatus, i.e., the encapsulation of allosteric effectors of hemoglobin in red blood cells by electroporation. It is to be understood that the apparatus may be adapted to accommodate other cell populations or vesicles, and other biologically active substances. Additionally, the apparatus maybe adapted to include other methods of encapsulation.

In accordance with the present invention, a sample of whole blood is admitted into the electroporation system **10** at input **11**.

Valve 12 is opened to admit the sample into the system 10. Simultaneously, valve 25 is opened and pump 22 is engaged to admit an anti-coagulant from the anti-coagulant reservoir 27. Valves 15 and 36 are also opened and pump 40 is engaged.

The admixture of anticoagulant and whole blood passes through a filter 18 and a pressure evaluation system 19, and is collected in a blood separation and wash bowl 44 which is activated when pump 40 is engaged. A sensor indicates when the blood separation and wash bowl 44 has been filled with red blood cells.

When pump 40 is engaged in a clockwise direction, the blood separation and wash bowl 44 is engaged and the anti-coagulant and whole blood suspension is centrifuged to separate the plasma, white blood cells, red blood cells, and waste. Valve 87 is opened to admit the plasma and white blood cells into the plasma reservoir 89.

Optionally, the cells retained in the separation and wash bowl 44 are then washed and centrifuged. Valves 33, 35, 15, and 36 are opened to admit saline buffer from the diluent reservoir 30 into the blood separation and wash bowl 44 which contains the red blood cells. Valve 12 is closed and pump 40 remains engaged.

During washing, valve 54 is opened to admit the waste into the waste reservoir 57 during the washing process. Again, the waste is stored in the waste reservoir 57 and the red blood cells are retained in the blood separation and wash bowl 44. The wash process is repeated if necessary. A contamination detection system may optionally be installed between the separation and wash bowl 44 and the waste reservoir 57 to control the wash process.

Following separation of the red blood cells, pump 40 is reversed, pump 22 is turned off, valves 12, 15, 33, 35, 36, 25, 87, and 54 are closed, and valve 97 is opened. If the cells were washed, pump 22 was previously turned off and valves 12 and 25 had been closed. The IHP solution is pumped out of the IHP reservoir 50 and into the separation and wash bowl 44 containing

the red blood cells. There, the red blood cells and IHP are admixed to form a suspension.

The preferred concentration of IHP in the solution is between approximately 10 mMol and 100 mMol with a more preferred concentration of approximately 23 to 35 mMol, and with a most preferred concentration of 35 mMol. The preferred IHP solution comprises the following compounds, in the following concentrations:

35 mMol IHP salt neutralized with 35 mMol IHP acid to a pH of 7.3
33 mMol K_2HPO_4
7 mMol NaH_2
30.6 mMol KCL
6.4 mMol NaCl
7.3 mMol Sucrose
5.0 mMol ATP

The IHP may be obtained from Sigma Chemical Company of St. Louis, Missouri.

The hematocrit of the suspension is preferably between approximately 30 and 60 with the most preferred hematocrit of approximately 50. Pump **40** is designed to pump both red blood cells and IHP solution and can be adjusted so that the final hematocrit entering the cooling coil **68** can be predetermined.

The steps of collecting the specimen, separating the cells from the specimen, washing the cells, and combining the cells with IHP can be done with an apheresis-blood washing machine, such as that manufactured by Haemonetics corporation. The apheresis-blood washing machine is coupled to the flow electrophoresis apparatus described herein to form a continuos flow electroporation apparatus.

After combining the red blood cells with the IHP solution, pump **40** is again reversed, valve **97** is closed and valve **64** is opened. The red blood cell-IHP suspension is then pumped through a thermoelectric cooling coil **68**. A blood bag from a

blood warming set, such as the blood bag provided in the Fenwal® Blood Warming Set manufactured by Baxter Healthcare Corporation can be used as the cooling coil **68**. When the red blood cell-IHP suspension passes through the cooling coil **68** in the cooling reservoir **69**, the suspension is cooled to a temperature of between approximately 1°C and 12°C, preferably approximately 4°C. Optionally, a pump may be added to the apparatus between the cooling coil **68** and cooling reservoir **69**, and the electroporation chamber **72**, to ensure a constant flow rate and compensate for fluctuation in volume that occurs when the cooling coil **68** is filled.

Optionally, the pre-cooling step may be eliminated and the red blood cell-IHP suspension may be directed to the electroporation chamber **72** immediately after admixing. In such an instance, the cooling coil **68** and cooling reservoir **69** would be eliminated from the continuos flow encapsulation apparatus **10**. Cooling prior to electroporation may not be required if the temperature of the electroporation chamber is sufficiently cool to maintain the cells suspension at 4°C.

After cooling, the red blood cell-IHP suspension enters the electroporation chamber **72**. The chamber **72** is maintained at a temperature of approximately 4°C. As the red blood cell-IHP suspension passes through the flow electroporation chamber **72**, an electric pulse is administered from a pulse generator **75** to the suspension causing openings to form within the cell membranes of the red blood cells.

The red blood cell-IHP suspension passes between two electrodes of the electroporation chamber **72**. Figs 3 to 10 describe the electroporation chamber. In a preferred embodiment of the present invention, when a suspension of non-treated cells enters the chamber **72**, the IHP-red blood cell suspension is subjected to approximately three high voltage pulses per volume at a fieldstrength of approximately 2600 to 3200 V/cm per pulse. The charge created across the cell membranes causes an electrical breakdown of the cell membrane, which

creates pores in the membrane. IHP then diffuses into the cell through these pores.

During electroporation, an electrical field of 1 to 3 KV/cm is created and maintained for a period of 1 to 4 milliseconds. The preferred pulse length is 3 to 4 milliseconds, with a most preferred pulse length of 2 milliseconds. Pulse length is defined as 1/e. At a flow rate of approximately 10.6 ml/minute, the preferred number of pulses is 3, at the preferred pulse rate of 0.29 Hz. The fieldstrength is defined as the voltage over the distance between the electrodes. The distance between electrodes is measured in centimeters. The preferred electrical parameters are as follows:

Exponential Pulse:	pulse length = 1.5 to 2.5 ms
	field strength = 2.7 to 3 KV/cm

Following electroporation, the red blood cell-IHP suspension enters an incubation chamber **78** where the suspension is incubated at room temperature for an incubation time of between approximately 10 minutes and 120 minutes with a preferred incubation time of 30 minutes. Optionally, the red blood cell-IHP suspension is incubated for approximately 5 minutes at a temperature of approximately 37°C, and at least 15 minutes at room temperature. The incubation chamber **78** may be surrounded by a heating means **80**. Any heating means **80** can be used in practicing the present invention. The preferred heating means **80** are a water bath or a thermoelectric heat pump.

Optionally, the incubator **78** contains a resealing buffer which aids in resealing and reconstitution of the red blood cells. In the preferred embodiment of the present invention, no resealing buffer is used.

Following incubation, the red blood cell-IHP suspension is returned to the blood separation and wash bowl **44** when valve **51** is opened and pump **40** is engaged. The excess IHP solution is removed from the red blood cell suspension by centrifugation. The waste IHP solution is directed to waste reservoir **57**. Valves

33, 37, 15 and 36 are then opened to admit a volume of post wash solution from reservoir 31 into the blood separation and wash bowl 44. In a preferred embodiment of the present invention, the post wash solution comprises a .9% $NaCl_2$ solution, including 2.0 mM $CaCl_2$ and 2.0 mM $MgCl_2$. Any physiological saline may be used.

After addition of the post wash solution, the red blood cell-IHP suspension is then centrifuged and the supernatant is discarded in the waste reservoir 57 through valve 54 leaving the red blood cells in the blood separation and wash bowl 44. The wash process is repeated until all unencapsulated IHP has been removed.

Optionally, as the waste is removed from the separation and wash bowl 44 it passes through a contamination detector 46 to detect any free IHP in the waste solution thereby confirming that exogenous non-encapsulated IHP has been removed from the modified red blood cells. The contamination detector 46 is optional.

Following washing, the red blood cells containing IHP may be reconstituted with the plasma and white blood cells retained in reservoir 89. Pump 40 is engaged and valves 87, 36, and 92 are opened. The modified red blood cells and plasma and white blood cells are pumped to reservoir 96. A filter may be installed between reservoir 96 and valve 92 to remove any aggregates or other impurities from the reconstituted modified blood.

The IHP-loaded red blood cells obtained in accordance with the method of the present invention can be administered directly back into the patient or the cells can be stored for later use. The IHP in the red blood cells is not released during the normal storage time.

It is contemplated that continuos flow encapsulation apparatus of the present invention may be modified to utilize other encapsulation methods.

Furthermore, it is contemplated that the continuous flow encapsulation apparatus may be adapted to process various diverse

cell populations. Furthermore, the apparatus may be used to encapsulate biologically active substances in artificial vesicles.

It is also contemplated that the continuos flow encapsulation apparatus of the present invention may be use to encapsulate a broad range of biologically active substances.

The flow electroporation apparatus of the present invention may be separated from the plasmaphoresis apparatus of the present invention. The blood cooling system, peristaltic pump, electroporation chamber, pulse generator, and electronics comprising the flow electroporation apparatus may be linked to a plasmaphoresis apparatus and interface with the controls of that machine.

While this invention has been described in specific detail with reference to the disclosed embodiments, it will be understood that many variations and modifications may be effected within the spirit and scope of the invention as described in the appended claims.

Flow Electroporation Chamber

During electroporation, the insertion rate of IHP is linearly dependent on the voltage administered to the cells. Generally, the higher the voltage, the more IHP is encapsulated; however, cell lysis is also increased and cell survival is decreased. The efficiency of an electroporation system may be judged by cell survival after electroporation. Poor cell survival indicates very low efficiency. The amplitude and duration of the electrical pulse is responsible for the electric breakdown of the cell membrane and creates pores in the pole caps parallel to the electric field. Thus, the factors to be considered in designing an electroporation system include the field strength, the pulse length and the number of pulses.

A perfect electroporation target is shaped like a sphere, so its orientation does not effect the efficiency of the applied field. When the target is spherical, a single pulse with an field strength above the threshold can electroplate 100 % of the target. Red blood cells are disk shaped. Because of their shape and

orientation in the electroporation chamber, only approximately 40 % of the cells are electroplated during a single pulse. To also electroporate the other 60 %, the fieldstrength can be increased. This increases the stress on the red blood cells in proper orientation to the electric field and leads to lower survival rates of the cells.

To achieve more efficient encapsulation while reducing the incidence of cell lysis and death, a flow electroporation chamber utilizing short duration multiple pulses was developed. With the flow-through rate steady and a steady field voltage, it was determined that plurality of pulses would insert maximal quantities of IHP with minimal 2 to 24 hour lysis. A multiple-pulse system allows an increase in the cell survival rate without increasing the field strength. When a multiple-pulse system is used, orientation of the cells is not as critical as it is when a system is a single pulse system is used. The lower field strength is much more gentle to the red blood cells. It is much easier to electroporate every single cell in the multiple pulse system, because the timing between the flow rate of the red blood cells through the chamber and the electroporation pulses, and the orientation of the cells is not as crucial as in a single pulse system. The flow multiple-pulse electroporation system also increases both the short term and the long term survival of red blood cells when compared to the single pulse method.

Figs. 11 to 13 illustrate the effects of various field strengths, under static or flow conditions, on the % oxygenation of IHP-encapsulated red blood cells over a range oxygen pressures; on the P_{50} value of IHP-encapsulated red blood cells (two concentrations of IHP solutions were compared); and, on the survival rates of red blood cells subjected to electroporation. All readings were taken 24 hours after electroporation. The results indicated that multiple pulses at comparatively low fieldstrengths produce optimal encapsulation results.

A cooled electroporation chamber is preferred to keep the red blood cells at a constant temperature during the electroporation process, thereby enhancing their survival rates.

This is accomplished by removing the excess heat created by the electrical pulse during the electroporation process. The excess heat may be removed either by cooling the electrodes or cooling the entire flow electroporation chamber. In accordance with the preferred embodiment of the present invention, the electrodes themselves are cooled.

During the electroporation process, blood is pumped through an inlet in the electroporation chamber and the red blood cells are subject to a series of electrical pulses as they travel through the chamber. They exit out the other end of the chamber. The chamber can be made of any type of insulating material, including but not limited to ceramic, teflon, plexiglass, glass, plastic, silicon, rubber or other synthetic materials. Preferably, the chamber is comprised of glass or polysulfone. Whatever the composition of the chamber, the internal surface of the chamber should be smooth to reduce turbulents in the fluid passing through it. The housing of the chamber should be non-conductive and biologically inert. In commercial use, it is anticipated that the chamber will be disposable.

In a preferred embodiment of the present invention, the electrodes that comprise part of the electroporation apparatus can be constructed from any type of electrically or thermally conductive hollow stock material, including but not limited to brass, stainless steel, gold plated stainless steel, gold plated glass, gold plated plastic, or metal containing plastic. Preferably, the surface of the electrode is gold plated. Gold plating serves to eliminate oxidation and reduces the collection of hemoglobin and other cell particles at the electrodes. The surface of the electrodes should be smooth.

The electrodes can be hollow, to allow cooling by liquid or gas, or the electrodes can be solid, to allow for thermoelectric or any other type of conductive cooling. Cooling could also be accomplished by cooling the electroporation chamber itself, apart from cooling the electrodes.

Preferably, the flow electroporation chamber is disposable. A detailed description of two embodiments of the electroporation chamber of the present invention is provided below.

In one embodiment, the flow electroporation chamber is constructed of clear polyvinyl chloride, and contains two opposing electrodes spaced a distance of approximately 7 mm apart. The electroporation chamber is a modification of a chamber obtained from BTX Electronic Company of San Diego, California. However, when the electroporation chamber is used continuously, it overheats and the survival rate of the cells processed by the apparatus decreases over time. To correct the overheating problem that occurred when the apparatus was used in a continuos flow manner, a continuous flow electroporation chamber was designed. A detailed description of the structure of the continuous flow electroporation chamber is provided below.

Figs. 3 through 8 show one embodiment of the flow electroporation chamber **72** of the present invention. As can be seen in Fig. 3, the flow electroporation chamber **72** includes a housing **100** having two electrodes **102** inset on opposing sides of the housing **100** of the electroporation chamber **72**. The housing **100** includes an inlet channel **104** at one end and an outlet channel **106** at the other. The inlet **104** and outlet **106** channels include connectors **108** and **109** respectively, preferably of the male Luer variety. The connectors **108** and **109** are hollow and form the inlet **104** and outlet **106** channels into the interior of the electroporation chamber **72**.

As seen in Figs 4 and 5, an internal chamber **110** extends most of the length of the housing **100** and is sized to receive the two electrodes **102**. The internal chamber **110** includes beveled surfaces **111** for receiving the internal edges of the electrodes **102**. The internal chamber **110** is thus formed by the internal surfaces of the electrodes **102** and the internal surfaces of the housing **100**. The internal chamber **110** is connected to the inlet **104** and outlet **106** channels.

As can be seen in Figs. 7 and 8, the electrodes **102** of the electroporation chamber **72** of Figs. 3 to 6 are comprised of flat,

elongated, hollow shells. The electrodes **102** include cooling inlets **112** and cooling outlets **114** at their ends. As described above, the rear surfaces of the electrodes **102**, or the surface to the left in Fig. 7, fits flush against the beveled surface **111** of the housing **100**.

The electroporation chamber **72** is designed such that the cell suspension to be subjected to electroporation enters the electroporation chamber **72** through the inlet **104** and expands to fill the internal chamber **110**. As the red blood cell suspension flows through the internal chamber **110** a pulse or charge is administered across the width of the internal chamber **110**.

To maintain a relatively constant temperature during the electroporation process, cooling fluid or cooling gas is pumped in the cooling inlet **112** and out the cooling outlet **114** so that the electrodes **102** are maintained at approximately 4° C.

Figs. 9 and 10 display a second embodiment of the flow electroporation chamber **172**. As can be seen in Figs. 9 and 10, the flow electroporation chamber **172** includes a hollow housing **200** substantially rectangular in shape. Two electrodes **202** are inserted into the interior of the housing **200** directly opposite one another, flush against the housing **200** walls. The flow electroporation chamber **172** further comprises an inlet channel **204** at one end and an outlet channel **206** at the other end of the housing **200**. The inlet **204** and outlet **206** channels include connectors **208** and **209** which are attached by tubing **216** to a cell suspension supply that supplies the cell suspension, i.e. the IHP-red blood cell suspension, to the electroporation chamber **172**. The connectors **208** and **209** and inlet **204** and outlet **206** channels serve to direct the cell suspension into and out of the housing **200**.

As can be seen in Fig. 10, one end of the inlet channel **204** and one end of the outlet channel **206** extends into the interior of the housing **200** forming an internal chamber **210**. The internal chamber **210** is thus formed by the internal surfaces of the electrodes **202**, the internal surfaces of the housing **200** and the internal surfaces of the of the inlet **204** and outlet **206** channels.

As can be seen in Figs. 9 and 10, the electrodes **202** of the flow electroporation chamber **172** comprise flat, elongated, hollow shells. The electrodes **202** include cooling inlets **212** and cooling outlets **214** at their ends, through which a gas or fluid may be pumped through the electrodes **202** to maintain a constant temperature during electroporation.. The electrodes **202** are connected to a pulse generator by cables **220.**

As with the chamber described above, the electroporation chamber **172** of Figs. 9 and 10 is designed such that the suspension to be subjected to electroporation enters the electroporation chamber **172** through the fluid inlet **204** and expands to fill the internal chamber **210**. As the red blood cells suspension flows through the internal chamber **210**, a pulse or charge is administered across the width of the internal chamber **210** between the electrodes **202.** To maintain a relatively constant temperature during the electroporation process, cooling fluid or cooling gas is pumped in the cooling inlet **212** and out the cooling outlet **214** of the electrodes **202** through the connectors **208** and **209** so that the electrodes **202** are maintained at approximately 4°C. It is also possible that the inlet channel **204**, outlet channel **206** and connectors **208** and **209** can be made as a solidly integrated glass part, rather than separate components.

It is contemplated that the flow electroporation chamber **172** maybe constructed from drawn glass or any other highly polished material. It is preferable that the interior surface of the electroporation chamber **172** be as smooth as possible to reduce the generation of surface turbulence. Drawn glass components are highly consistent with perfect surface finishes. Furthermore, they are stable and inert to blood components. They are also relatively inexpensive, which is desirable for a disposable electroporation chamber.

The electrodes may also be comprised of drawn glass, electroplated with colloidal gold. Again, the surfaces of the electrodes should be highly finished, highly conductive, yet biologically inert. Gold electroplate is durable and inexpensive.

Fluidic connection can be accomplished using commonly available parts.

The flow electroporation chamber may be constructed either as a part of the entire flow encapsulation apparatus, or as an individual apparatus. The flow electroporation apparatus may then be connected to a commercially available plasmaphoresis machine for encapsulation of particular cell populations. For example, the flow electroporation chamber maybe connected to commercially available plasmaphoresis equipment by electronic or translational hardware or software. Optionally, a pinch-valve array and controller driven by a PC program can also be used to control the flow electroporation apparatus. Similarly, current power supplies are capable of establishing the power levels needed to run the flow electroporation chamber or flow encapsulation apparatus.

While this invention has been described in specific detail with reference to the disclosed embodiments, it will be understood that many variations and modifications may be effected within the spirit and scope of the invention as described in the appended claims.

Application of IHP treated red blood cells

The present invention provides a novel method for increasing the oxygen-carrying capacity of erythrocytes. In accordance with the method of the present invention, the IHP combines with hemoglobin in a stable way, and shifts its oxygen releasing capacity. Erythrocytes with IHP-hemoglobin can release more oxygen per molecule than hemoglobin alone, and thus more oxygen is available to diffuse into tissues for each unit of blood that circulates. Under ordinary circumstances, IHP is toxic and cannot be tolerated as an ordinary drug. Attachment of IHP to hemoglobin in this novel procedure, however, neutralizes its toxicity. In the absence of severe chronic blood loss, treatment with a composition prepared in accordance with the present method could result in beneficial effects that persist for approximately ninety days.

Another advantage of IHP-treated red blood cells is that they do not lose the Bohr effect when stored. Normal red blood cells that have been stored by conventional means do not regain their maximum oxygen carrying capacity for approximately 24 hours. This is because the DGP in normal red blood cells diffuses away from the hemoglobin molecule during storage and must be replaced by the body after transfusion. In contrast, red blood cells treated according to the present invention are retain their maximum oxygen carrying capacity during storage and therefore can deliver maximum oxygen to the tissues immediately after transfusion into a human or animal.

The uses of IHP-treated RBC's is quite extensive including the treatment of numerous acute and chronic conditions including, but not limited to, hospitalized patients, cardiovascular operations, chronic anemia, anemia following major surgery, coronary infarction and associated problems, chronic pulmonary disease, cardiovascular patients, autologous transfusions, as an enhancement to packed red blood cells transfusion (hemorrhage, traumatic injury, or surgery). congestive heart failure, myocardial infarction (heart attack), stroke, peripheral vascular disease, intermittent claudication, circulatory shock, hemorrhagic shock, anemia and chronic hypoxmia, respiratory alkalemia, metabolic alkalosis, sickle cell anemia, reduced lung capacity caused by pneumonia, surgery, pneumonia, trauma, chest puncture, gangrene, anaerobic infections, blood vessel diseases such as diabetes, substitute or complement to treatment with hyperbaric pressure chambers, intra-operative red cell salvage, cardiac inadequacy, anoxia - secondary to chronic indication, organ transplant, carbon monoxide, nitric oxide, and cyanide poisoning.

Treating a human or animal for any one or more of the above disease states is done by transfusing into the human or animal between approximately 0.5 and 6 units (1 unit = 500 ml) of IHP-treated blood that has been prepared according to the present invention. In certain cases, there may be a substantially complete replacement of all the normal blood in a patient with

IHP-treated blood. The volume of IHP-treated red blood cells that is administered to the human or animal will depend upon the indication being treated. In addition, the volume of IHP-treated red blood cells will also depend upon concentration of IHP-treated red blood cells in the red blood cell suspension. It is to be understood that the quantity of IHP red blood cells that is administered to the patient is not critical and can vary widely and still be effective.

IHP-treated packed RBC's are similar to normal red blood cells in every category except that the IHP-treated packed red blood cells can deliver 2 to 3 times as much oxygen to tissue per unit. A physician would therefore chose to administer a single unit of IHP-treated packed red blood cells rather than 2 units of the normal red blood cells. IHP-treated packed red blood cells could be prepared in blood processing centers analogously to the present blood processing methods, except for the inclusion of a processing step where the IHP is encapsulated in the cells.

While this invention has been described in specific detail with reference to the disclosed embodiments, it will be understood that many variations and modifications may be effected within the spirit and scope of the invention as described in the appended claims.

What is claimed is:

1. A closed continuous flow apparatus for encapsulating a biologically-active substance into red blood cells comprising:

a. introduction means for introducing blood into the apparatus;

b. separating means in closed fluid communication with the introduction means for providing a red blood cell fraction;

c. mixing means in closed fluid communication with the separating means for mixing the red blood cells with an effective amount of a solution of a biologically-active substance to provide a red blood cell suspension;

d. electroporation means in closed fluid communication with the mixing means for subjecting the red blood cell suspension to electroporation thereby causing the biologically-active substance to be encapsulated in the red blood cells;

e. incubating means in closed fluid communication with the electroporation means for incubating the red blood cells after electroporation to provide modified red blood cells; and

f. washing means in closed fluid communication with the incubating means for washing the modified red blood cells to remove unencapsulated biologically-active substance therefrom.

2. The apparatus of Claim 1, further comprising a cooling means for cooling the red blood cell suspension after element (c).

3. The apparatus of Claim 1, further comprising a means for heating the incubating means.

4. The apparatus of Claim 1, further comprising a detection means for detecting extracellular biologically-active substance after element (f).

5. The apparatus of Claim 1, wherein the separating means is capable of separating the plasma and leukocyte fraction from the red blood cell fraction.

6. The apparatus of Claim 1, further comprising a collecting means in closed fluid communication with the washing means and in closed fluid communication with the holding means for combining the modified red blood cells with the plasma and leukocytes after element (f).

7. The apparatus of Claim 1, wherein the biologically-active substance is inositol hexaphosphate.

8. A method of incorporating a biologically-active substance into a red blood cell in a closed continuous flow system comprising the steps of:

a. introducing blood into the closed continuous flow system;

b. separating the components of the blood to provide a plasma and leukocyte fraction and a red blood cell fraction;

c. mixing the red blood cells with an effective amount of a biologically-active substance to provide a red blood cell suspension;

d. electroporating the red blood cell suspension thereby causing the biologically-active substance to be encapsulated in the blood cells;

e. incubating the modified red blood cells to allow the modified red blood cells to reseal; and

f. washing the modified red blood cells to remove unencapsulated biologically-active substance therefrom.

9. The method of Claim 8, further comprising the step of cooling the red cell suspension before electroporating the red cell suspension.

10. The method of Claim 8, further comprising the step of heating the red blood cell suspension during the incubating step.

11. The method of Claim 8, further comprising the step of detecting extracellular biologically-active substance after step (f).

12. The method of Claim 8, further comprising the step of separating the plasma and leukocyte fraction from the red blood cell fraction and storing the plasma and leukocyte fraction.

13. The method of Claim 8, further comprising the step of restoring plasma and leukocytes to the modified red blood cells after step (f).

14. The method of Claim 8, wherein the biologically-active substance is inositol hexaphosphate.

15. A closed continuous flow apparatus for encapsulation of a biologically-active substances into cells by electroporation comprising:

a. introduction means for introducing the cells into the apparatus;

b. separating means in closed fluid communication with the introduction means for isolating the cells;

c. mixing means in closed fluid communication with the separating means for mixing the cells with an effective amount of a solution of a biologically-active substance to provide a cell suspension;

d. electroporation means in closed fluid communication with the mixing means for subjecting the cell suspension to electroporation thereby causing the biologically-active substance to be encapsulated in the cells;

e. incubating means in closed fluid communication with the electroporation means for incubating the cells after electroporation to modified cells; and

f. washing means in closed fluid communication with the incubating means for washing the modified cells to remove unencapsulated biologically-active substance therefrom.

16. The apparatus of Claim 15, further comprising a cooling means for cooling the cell suspension after element (c).

17. The apparatus of Claim 15, further comprising a means for heating the incubating means.

18. The apparatus of Claim 15, further comprising a detection means for detecting unencapsulated biologically-active substance after element (f).

19. The apparatus of Claim 15, further comprising a collecting means in closed fluid communication with the washing means and in closed fluid communication with the holding means for storing the washed, modifed cells after element (f).

20. A method of incorporating a biologically-active substance into a cell in a closed continuous flow system comprising the steps of:

a. introducing the cells into the closed continuous flow system;

b. isolating the cells;

c. mixing the cells with an effective amount of a biologically-active substance to provide a cell suspension;

d. electroporating the cell suspension thereby causing the biologically-active substance to be encapsulated in the cells;

e. incubating the cells to allow the modified cells to reseal; and

f. washing the modified cells to remove unencapsulated biologically-active substance therefrom.

21. The method of Claim 20, further comprising the step of cooling the cell suspension before electroporating the cell suspension.

22. The method of Claim 20, further comprising the step of heating the cell suspension during the incubating step.

23. The method of Claim 20, further comprising the step of detecting extracellular biologically-active substance after step (f).

24. The method of Claim 20, further comprising the step of collecting the modified cells after step (f).

25. A composition prepared in accordance with the method of Claim 20.

26. A composition prepared in accordance with the method of Claim 8.

27. A composition prepared in accordance with the method of Claim 14.

28. A method of treating a disease comprising the step of administering to a human or animal a suspension of red blood cells treated according to Claim 8.

29. A device comprising:

a continuous internal chamber extending therethrough, the internal chamber defining internal walls and an input channel and an output channel, the input channel of the internal chamber configured to receive a continuous flow of blood which flows through the internal chamber and through the output channel;

a first electrode extending along a specified length of one side of the internal chamber; and

a second electrode extending along a specified length of another side of the internal chamber, the first and second electrodes being designed to emit pulses of electronic energy from the first electrode through the blood as the blood flows through the internal chamber to the second electrode;

the number of pulses of electricity through the electrodes per minute, the specified length of the first electrode, the specified length of the second electrode, and the volume of blood flowing through the internal chamber being such that the continuous flow volume of blood may be electroporated while flowing through the internal chamber.

30. The device of Claims 29, wherein the first electrode and the second electrode each form at least part of the internal wall of the internal chamber.

31. The device of Claim 29, wherein the first electrode includes an internal chamber along its length, the internal chamber of the first electrode defining an input and an output, the input of the first electrode configured to receive a coolant which flows through the internal chamber of the first electrode and through the output of the first electrode.

32. The device of Claim 29, wherein the second electrode includes an internal chamber along its length, the internal chamber defining an input and an output, the input of the second electrode configured to receive a coolant which flows through the internal chamber of the second electrode and through the output of the second electrode.

33. A method of electroporating blood comprising the steps of:

providing a device comprising:

a continuous internal chamber extending through, the internal chamber defining internal walls and an input and an output, the input of the internal chamber configured to receive a continuous flow of blood which flows through the internal chamber and through the output;

a first electrode extending along a specified length of one side of the internal chamber; and

a second electrode extending along a specified length of another side of the internal chamber, the first and second electrodes being designed to emit pulses of electronic energy from the first electrode through the blood as the blood flows through the internal chamber to the second electrode;

introducing a continuous flow volume of blood into the input of the internal chamber, through the internal chamber and out of the output of the internal chamber at a specified flow volume; and

simultaneously emitting a continuous, pulsating electronic current from the first electrode through the blood to the second electrode.

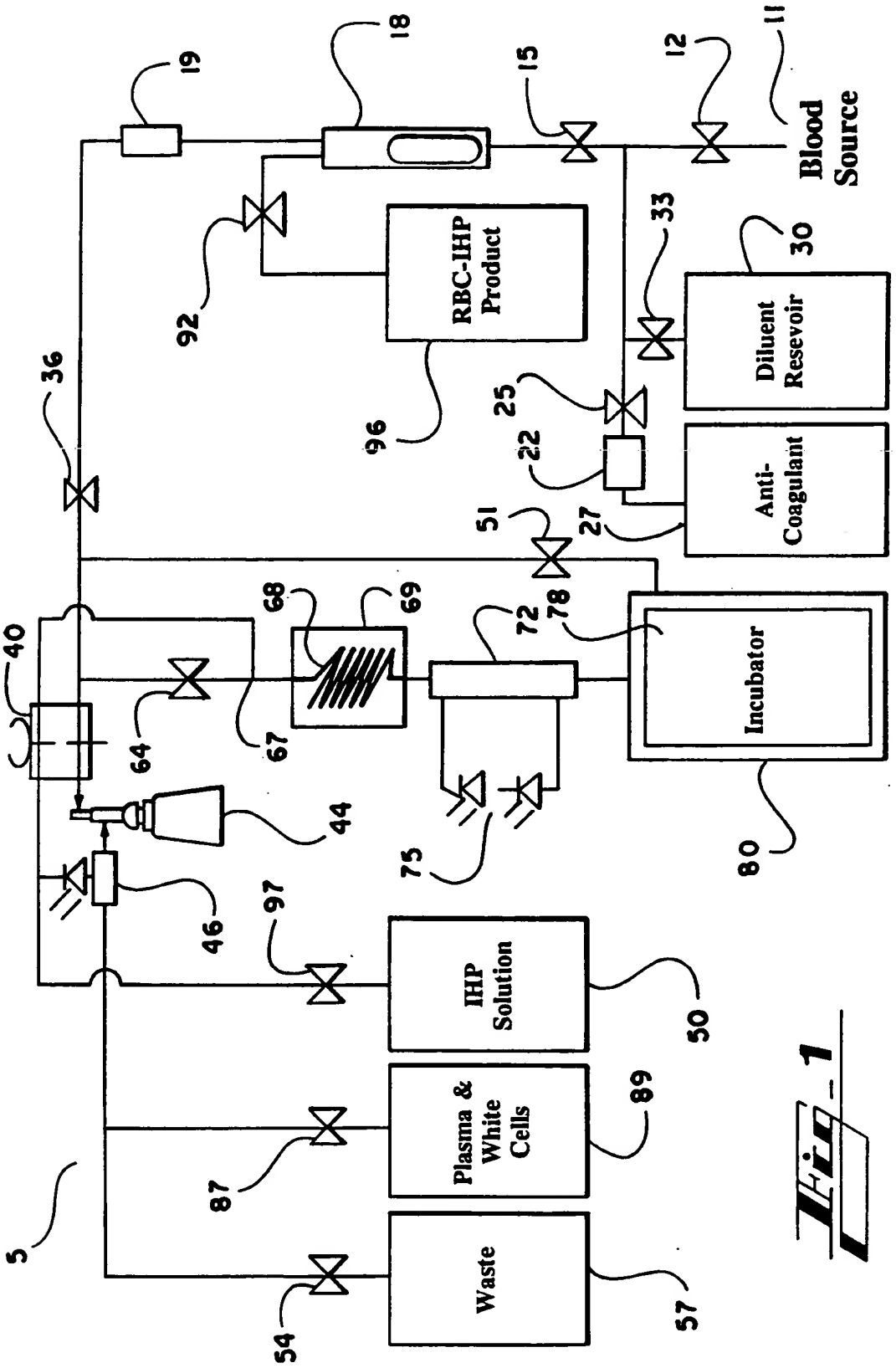

Blood Source
RBC-IHP Product
Diluent Resevoir
Anti-Coagulant
Incubator
IHP Solution
Plasma & White Cells
Waste
11
12
15
18
19
22
25
27
30
33
36
40
44
46
50
51
54
57
64
67
68
69
72
75
78
80
87
89
92
96
97
5

Fig. 1

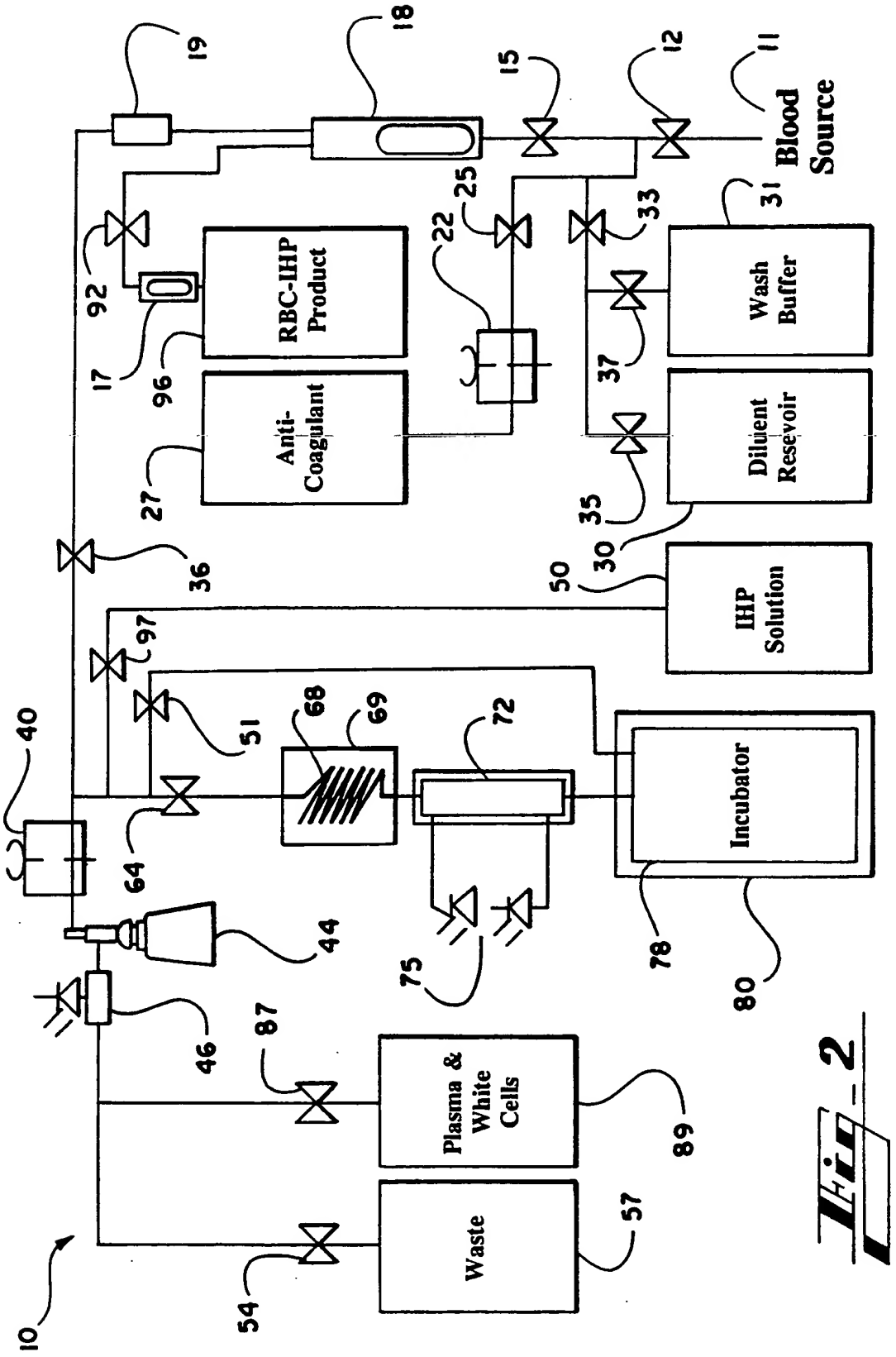

Blood Source
RBC-IHP Product
Anti-Coagulant
Wash Buffer
Diluent Reservoir
IHP Solution
Incubator
Plasma & White Cells
Waste
10
11
12
15
17
18
19
22
25
27
30
31
33
35
36
37
40
44
46
50
51
54
57
64
68
69
72
75
78
80
87
89
92
96
97

Fig. 2

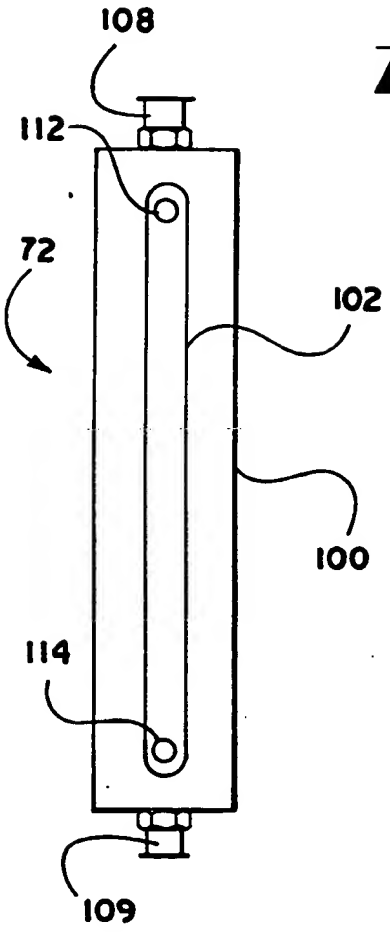


Fig_3

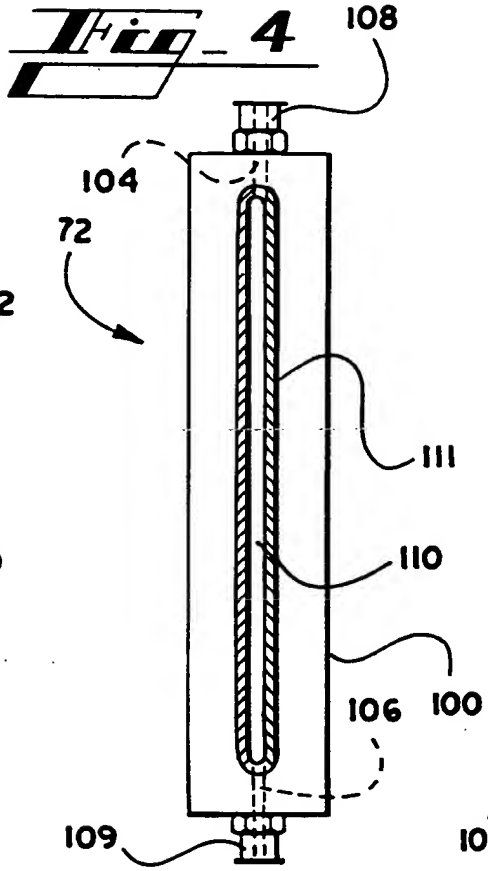


Fig_4

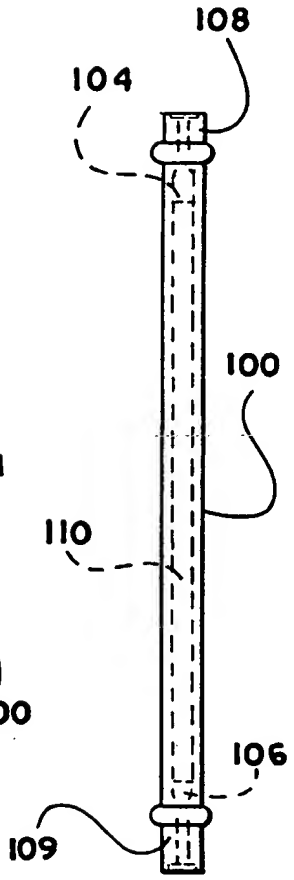


Fig_5

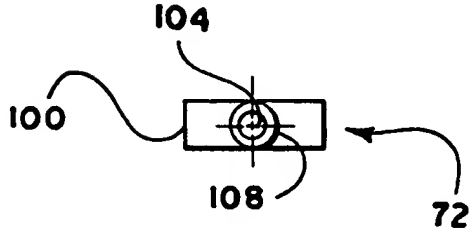


Fig_6

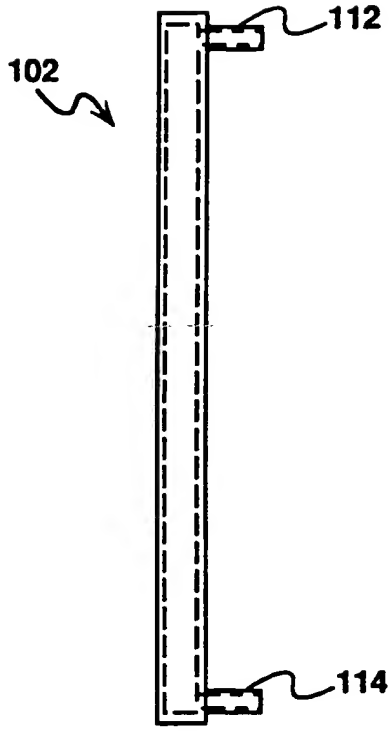


Fig. 7

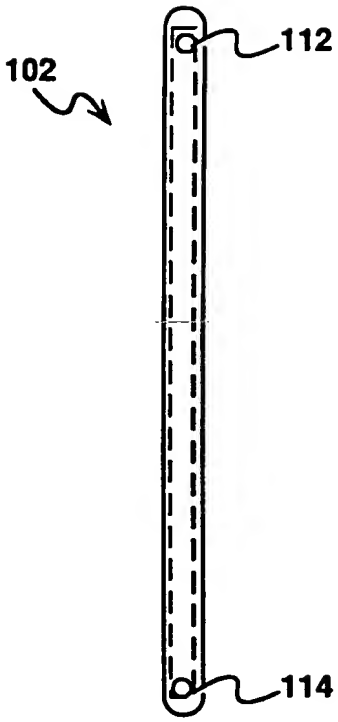


Fig. 8

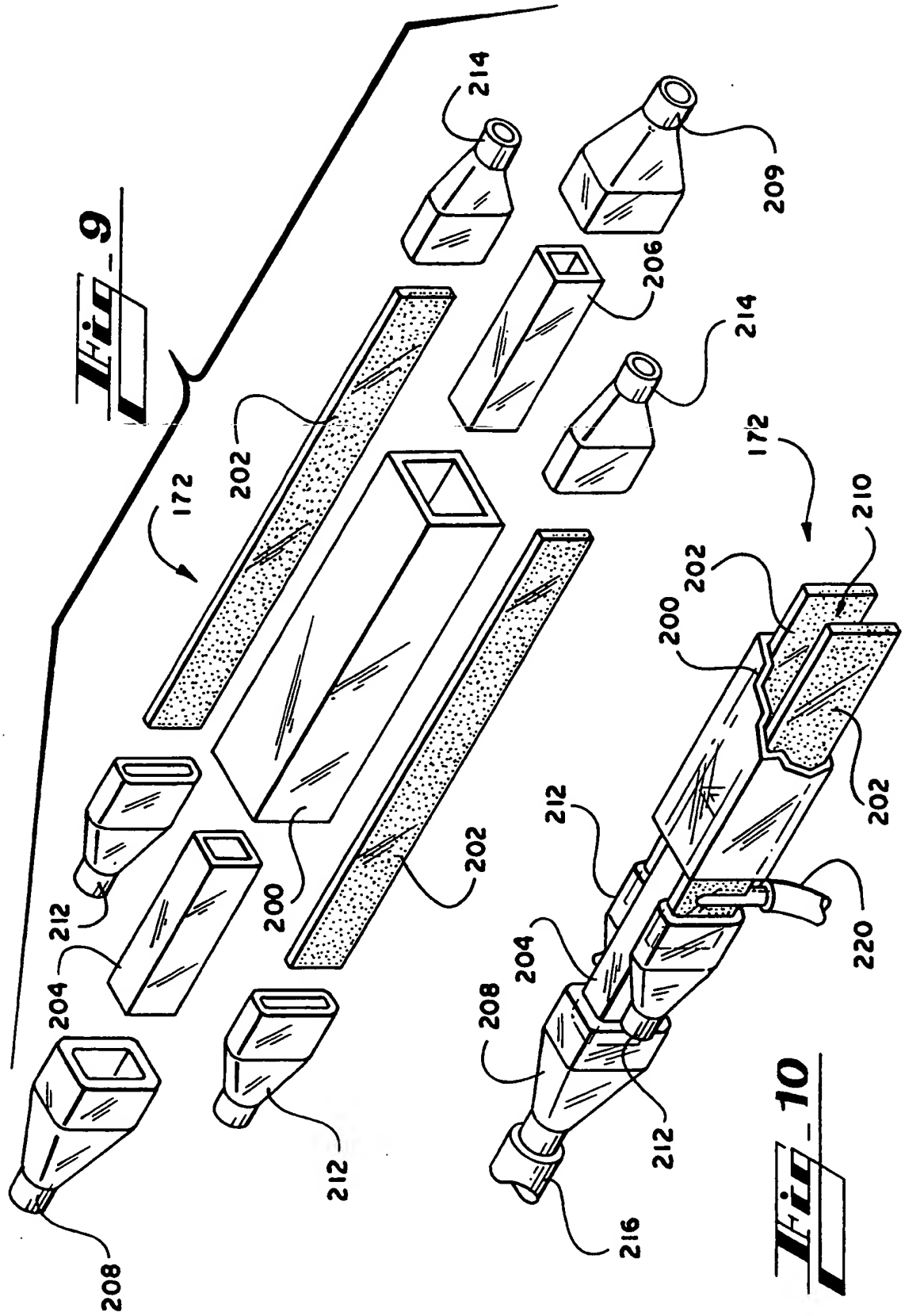

214
209
206
214
172
202
210
200
202
202
212
202
200
212
204
220
208
204
212
208
216

Fig_9

Fig_10

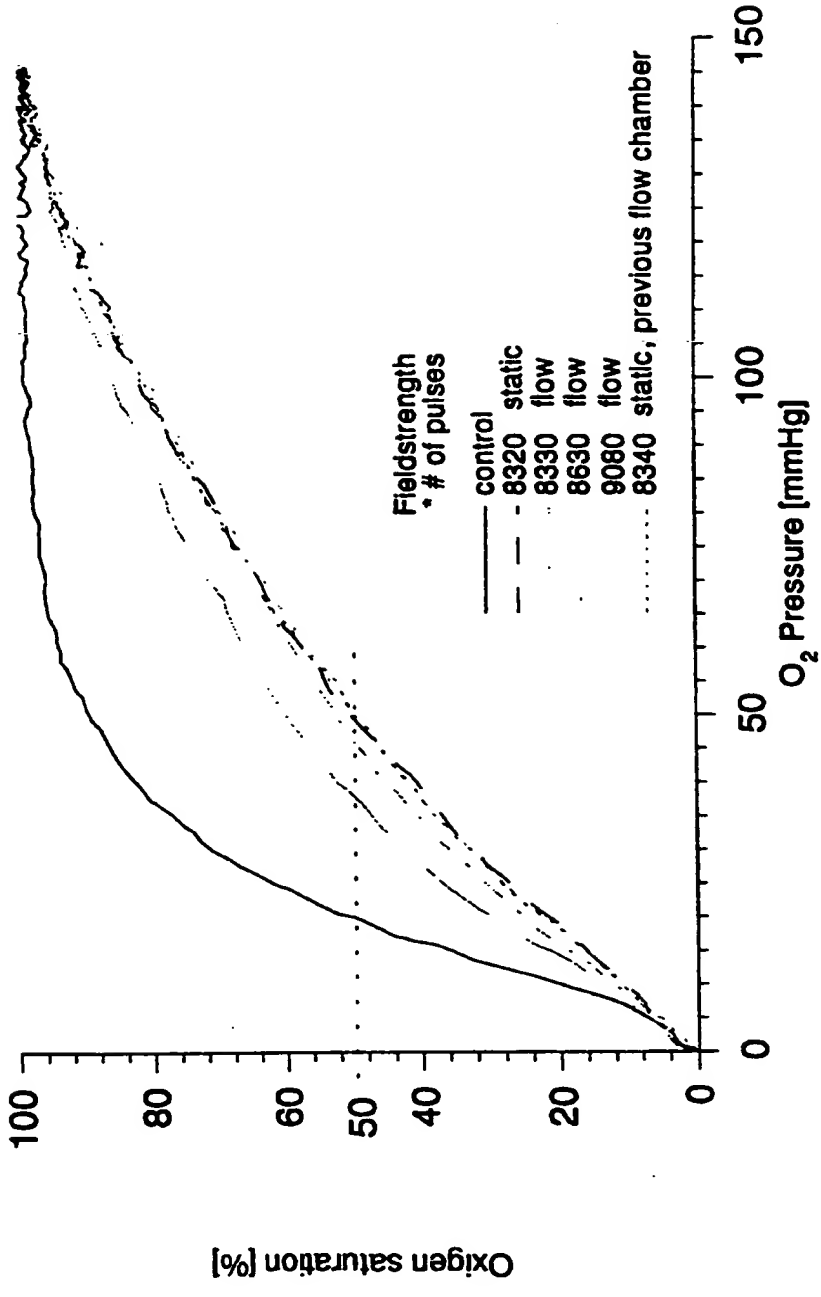

Oxigen saturation [%]
100
80
60
50
40
20
0
0
50
100
150
O_2 Pressure [mmHg]
Fieldstrength
* # of pulses
control
- 8320 static
8330 flow
8630 flow
9080 flow
8340 static, previous flow chamber

Fig. 11

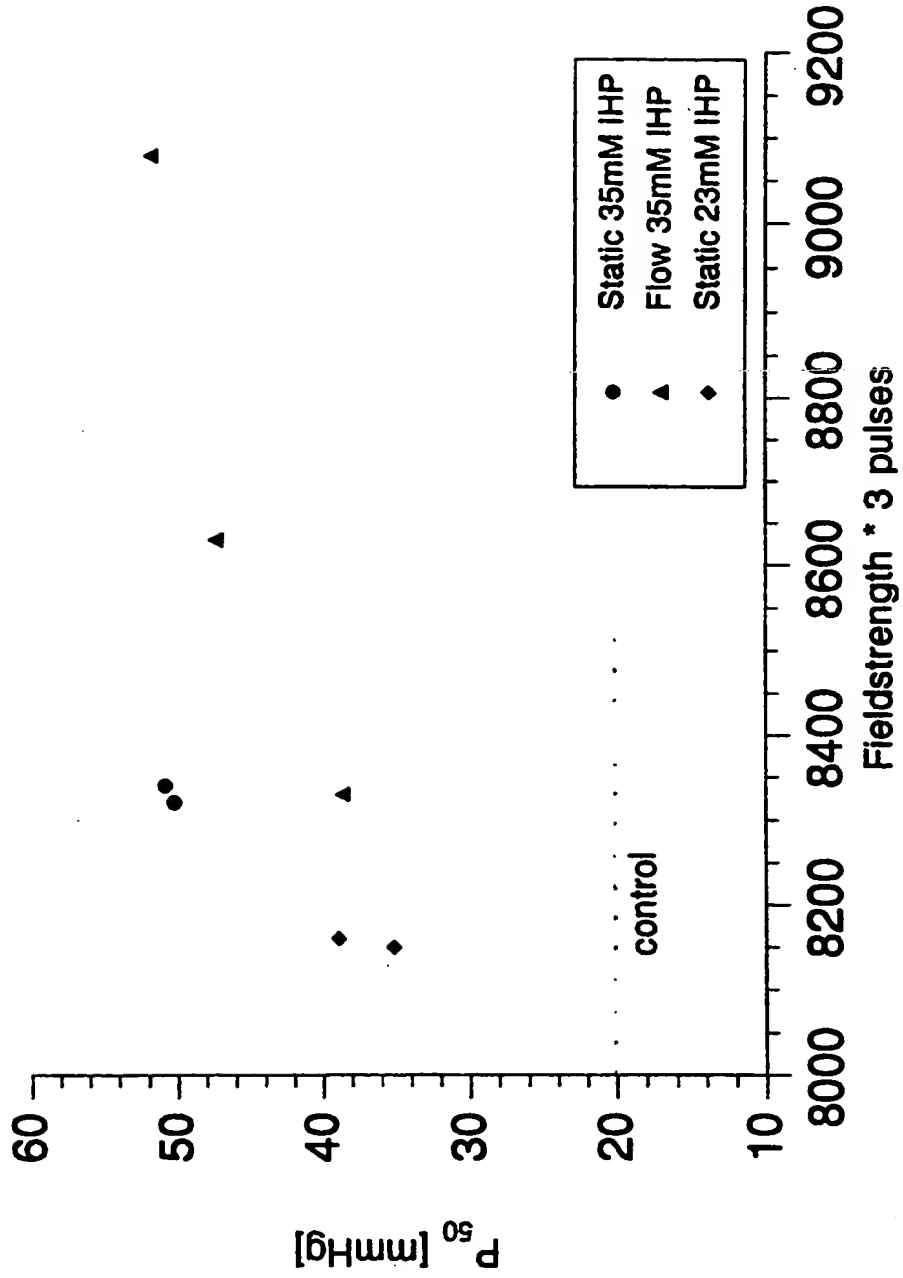

P50 [mmHg]
60
50
40
30
20
10
8000
8200
8400
8600
8800
9000
9200
Fieldstrength * 3 pulses
control
Static 35mM IHP
Flow 35mM IHP
Static 23mM IHP

Fig. 12

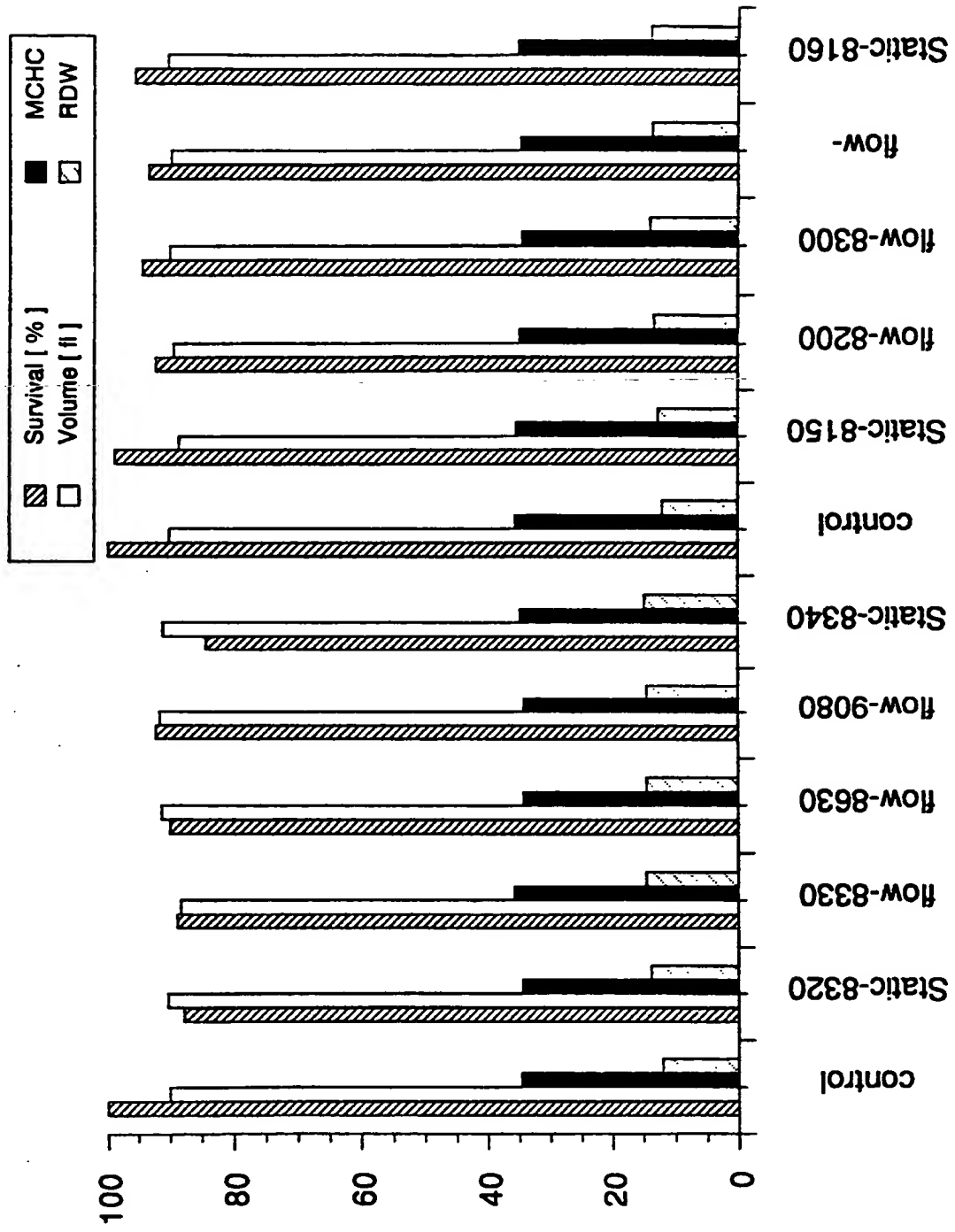

Survival [%]
Volume [fl]
MCHC
RDW
100
80
60
40
20
0
control
Static-8320
flow-8330
flow-8630
flow-9080
Static-8340
control
Static-8150
flow-8200
flow-8300
flow-
Static-8160

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03189

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog: Biosis, Medline, EmBase
search terms: erythrocyte?, electorporat?, inositol hexaphosphate, allosteric inhibit?, hemoglobin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,652,449 (ROPARS ET AL) 24 MARCH 1987, see col.2, lines 55-61; col. 3, lines 8-29; col. 5, lines 5-10; col. 9, lines 48-50; col. 11, lines 8-20 and 42-47; col. 16, lines 5-68; col. 18, lines 28-32.	1-32
Y	FEBS LETTERS, Volume 275, Number 1, 2, issued November 1990, Y. Mouneimne, "Stable Rightward Shifts of the Oxyhemoglobin Dissociation Curve Induced by Encapsulation of Inositol Hexaphosphate in Red Blood Cells Using Electroporation", pages 117-120, see page 117, col. 2, lines 11-24; page 118, col. 1, lines 14-18; page 119, col. 2, lines 5-18.	1-32
Y	US, A, 4,800,163 (HIBI ET AL) 24 JANUARY 1989, see col. 2, lines 1-10.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 APRIL 1994	Date of mailing of the international search report MAY 13 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. GROMET Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03189

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,321,259 (NICOLAU ET AL) 23 MARCH 1982, see col. 8, lines 14-16.	4,11,18,23
Y	US, A, 5,135,667 (SCHOENDORFER) 04 AUGUST 1992, see col. 1, lines 21-31.	5-6, 12-13, 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03189

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01N 1/02, 63/00; A61K 37/02; B01D 61/42; C12M 1/00, 1/02, 1/12, 1/36; C12N 5/16, 13/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

204/131, 180.1, 183.1, 274, 275, 299R, 403; 210/645; 424/93U, 93V, 93AA, 533;
435/2, 173, 240.1, 240.2, 287, 289, 311, 316

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

204/131, 180.1, 183.1, 274, 275, 299R, 403; 210/645; 424/93U, 93V, 93AA, 533;
435/2, 173, 240.1, 240.2, 287, 289, 311, 316